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Going Beyond Phosphorylation: Acetylphosphate-Mediated Ran Polymerase Acetylation and Caper Phosphorylation Co-Regulate Transcription of Escherichia Coli Stress-Responsive Gene cpxP

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GOING BEYOND PHOSPHORYLATION:

ACETYLPHOSPHATE-MEDIATED

RNA POLYMERASE ACETYLATION AND CPXR PHOSPHORYLATION

CO-REGULATE TRANSCRIPTION

OF *ESCHERICHIA COLI* STRESS-RESPONSIVE GENE *CPXP*.

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The important thing is not to stop questioning. Curiosity has its own reason for existing.

—Albert Einstein

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ABSTRACT

The ability of bacteria to sense and adapt to environmental changes has allowed these organisms to thrive in all parts of the globe and to establish many complex interactions with the environments in which they live as well as with other members of these environments. Crucial to monitoring extracellular conditions is a group of signaling pathways known as two-component signal transduction systems (2CST). These systems relay information from the extracellular environment to the interior of the cell via a transfer of phosphoryl groups from the sensory protein known as a sensor kinase (SK) to the output protein known as a response regulator (RR).

In addition to this constant input of extracellular information, bacterial cells also monitor their intracellular environment, as among other things, it provides precious information about their nutritional status. It is the integration of these extracellular and intracellular cues that will determine which genes should be transcribed, assuring the most appropriate response for each environment at any given time.

Here, I present evidence that one of these 2CST systems in *E. coli*, the CpxAR system, integrates signals originated from both the extracellular environment and from central metabolic pathways to modulate transcription of the CpxR-dependent

gene *cpxP*. Through a series of genetic and biochemical experiments I demonstrate that acetyl-phosphate (AcP), a central metabolite originated from the Pta-AckA pathway, donates its phosphoryl group to the RR CpxR both *in vitro* and *in vivo* and that this transfer of phosphoryl group activates *cpxP* transcription.

In addition to CpxR phosphorylation, my data suggest that *cpxP* transcription is also sensitive to an additional posttranslational modification, N^{ε}-lysine acetylation of RNA polymerase (RNAP). My data implicate the acetylation of two lysine side chains on the carboxyl terminal domain of the α subunit (αCTD) of RNAP in the modulation of *cpxP* transcription in response to the AcP-dependent phosphorylation of CpxR. Together, CpxR phosphorylation and the acetylation of Lys-298 on αCTD contribute to transcriptional activation of *cpxP*, whereas acetylation of Lys-291 dampens this response. Thus, protein phosphorylation and protein acetylation, seem to act together to fine-tune *cpxP* transcription.

CHAPTER ONE

INTRODUCTION

Bacteria are ubiquitous organisms. Their wide spread prevalence plays an important role in the diverse environments they inhabit. In the soil, bacteria are key contributors to the nitrogen cycle, and different steps of this cycle are carried out by different groups of bacteria [reviewed in (15)]. In the ocean, bioluminescent bacteria are thought to help the Hawaiian bobtail squid escape predation by providing light that the squid uses to counter illuminate its shadow [reviewed in (62)]. In mammals, gut bacteria have been shown to contribute to host metabolism, potentially contributing to obesity and diabetes (13, 134). The complex and diverse microbiota in the human gut has been shown to interact with several branches of the immune system, influencing gut barrier and defense mechanisms. It is thought that these interactions influence immune homeostasis and that an imbalance in this homeostasis could contribute to autoimmune diseases (22, 65). Additionally, the human intestinal microbiota is thought to play an important role in preventing the establishment of opportunistic infection by preventing the establishment of invading species and/or by helping control the number of some potentially harmful members of the community (3).

These different interactions with their environment are the result of many years of coevolution (105), resulting in an intricate and sophisticated interaction among the different species involved. These interactions often require that different organisms can sense each other and their environment, and that they can quickly respond to changing conditions, assuring that the most appropriate genes and proteins are transcribed and synthesized at the precise amount at any given time.

To sense changes to the extracellular environment, bacteria commonly use sensory systems known as two-component signal transduction (2CST) pathways. As the name implies, the simplest of these pathways consists of only two components: a sensor kinase (SK) and a response regulator (RR) (**Fig.1 Left**). The SK is a histidine kinase that autophosphorylates a conserved histidine (His) residue using ATP as its phosphoryl donor. Once phosphorylated, the SK serves as the phosphoryl donor to its cognate RR, an aspartyl kinase that autophosphorylates a conserved aspartate (Asp) residue located in its receiver domain [reviewed in (127)]. In general, the more complex the environment in which a bacterial species lives, the greater the number of 2CST pathways encoded by its genome (16).

In addition to constantly monitoring their extracellular environment, bacteria must also closely monitor their intracellular environment, as such monitoring provides important information concerning nutritional status. This nutritional information allows a bacterial cell to 'decide' how much energy it can invest in different physiological processes and ultimately decide whether the cell should remain metabolically active or become quiescent (61).

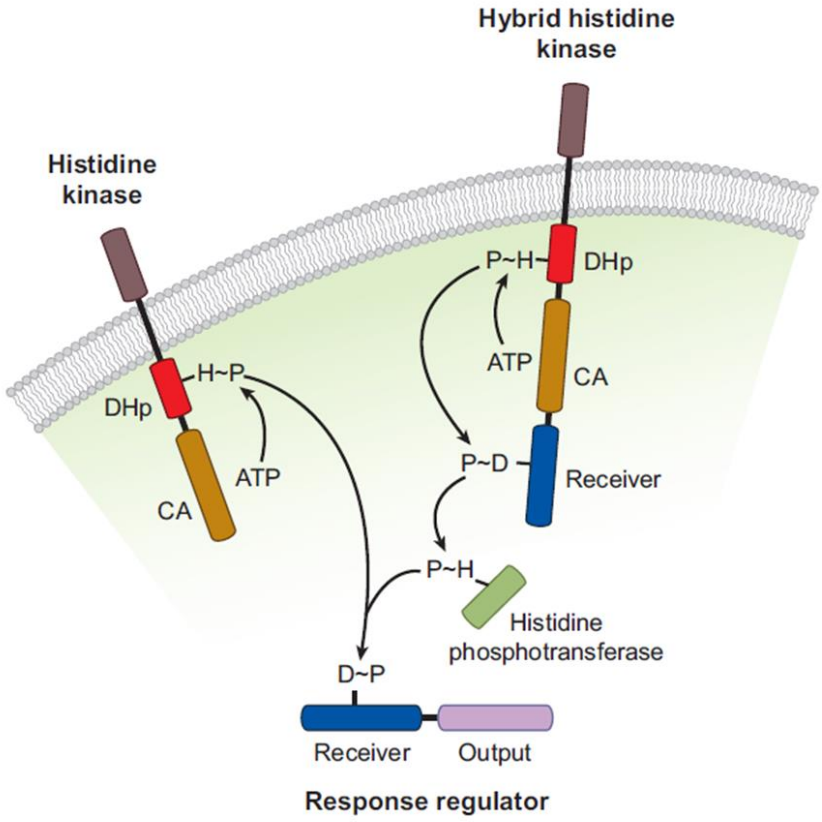


Fig. 1 Schematic overview of the 2CST. (left) The prototypical 2CST with its catalytic and ATPase (CA) responsible for binding ATP and catalyzie an utophosphorylation of a conserved histidine found within the dimerization and histidine phosphotransferase (DHp) domain. The DHp domain serves as the phosphodonor for a cognate response regulator which typically contain two domains, a receiver domain and an output domain. Receiver domains contain the phosphoacceptor aspartate and several other highly conserved amino acids that catalyze phosphotransfer from a histidine kinase. Output domains, which are activated by phosphorylation of the receiver domain, are varied, but are often involved in binding DNA. Phosphorelays or hybirids 2CST (*right*) are a common variant of the two-component signaling paradigm. Receipt of a stimulus activates autophosphorylation of a hybrid histidine kinase. The phosphoryl group is then passed intramolecularly to a C-terminal receiver domain, similar to that found in response regulators. A histidine phosphotransferase (HPT) then shuttles the phosphoryl group from the hybrid kinase to a soluble response regulator containing an output domain.

The cells can receive cues concerning their nutritional status by monitoring the ratios and/or concentrations of important molecules, such as NAD^+/NADH, ATP/ADP, cAMP and ppGpp. The information obtained from the ratios and/or concentrations of these different molecules must be integrated with signals that are being received from the extracellular environment. Proper signal integration should promote the most adequate amount of expression and activity of the proteins most suited for survival at that specific environmental condition. One of the many ways by which bacterial cells can integrate large amounts of constant and diverse sensory information is posttranslational modification of proteins, especially of proteins involved in signal transduction and proteins involved in the transcriptional machinery that convert the signal into action.

My dissertation focuses on the mechanism by which one signaling pathway of *Escherichia coli,* the CpxAR 2CST system, integrates intracellular cues to regulate the *cpxP* promoter, which drives expression of a chaperone that respond to periplasmic stresses, such as unfolded proteins. The data I will provide here supports a model in which the CpxAR pathway utilizes intracellular information to modulate *cpxP* transcription via an acetylphosphate (AcP)-dependent phosphorylation of the RR CpxR and AcP-dependent acetylation of RNA polymerase (RNAP).

During the next few pages, I will provide a brief overview of 2CST and the CpxAR pathway. I will then provide some background information on AcP and Acetyl-Coenzyme A (AcCoA), followed by an overview of the current understanding of

protein acetylation. I will end with an explanation of RNAP and the steps involved in transcription initiation.

TWO-COMPONENT PATHWAYS

Two-component signal transduction (2CST) pathways are sophisticated sensory systems found in bacteria, archaea and a few eukaryotic organisms (127). These systems help organisms sense and mount a response to changes in their environment. The classic 2CST pathway contains only two proteins, a sensor kinase protein (SK) and a cognate response regulator (RR) (**Fig. 1 left**). The SK is often but not always integral to or associated with the cellular membrane (12). The RR is often but not always involved in transcriptional regulation and thus often but not always will contain a DNA-binding domain (41, 42). 2CST proteins are modular and, in general, RRs possess two independent domains while SKs contain two highly conserved domains, a dimerization and histidine phosphotransfer (DHp) domain and the catalytic and ATP binding (CA) domain, and at least one and sometimes several additional domains (40). 2CST pathways also can be made of more than two proteins (**Fig. 1 right**), adding complexity to the transfer of information from the SK to the RR as it increases the number of domains and/or proteins that are involved in the signal transduction; thus, increasing the number of places on the pathway in which the transfer of information can be regulated (12). For the purpose of this dissertation, henceforth, I will focus on the less complex and more orthodox form of these 2CST pathways.

Many organisms encode multiple 2CST that are involved in sensing different environmental signals. In general, 2CST pathways share a large degree of homology among each other. Thus, many mechanisms are in place to prevent an erroneous transduction of signal between non-cognate 2CST pathways, also known as cross-talk. In order to achieve this specificity, cognate SKs and RRs are thought to have co-evolved to incorporate specificity residues along the surface or regions used for their interaction. These residues decrease the rate of phosphoryl transfer among non-cognate proteins, while maintaining a high transfer rate among cognate proteins [reviewed in (100)].

The relay of information from the SK to the RR, leading to the final output, requires multiple phosphoryl transfers. It initiates with the transfer of phosphoryl groups from the γ phosphoryl group of ATP to a conserved His side chain on the SK. This transfer of phosphoryl group from ATP to the conserved His side chain of the SK is mediated by conserved residues on the ATP-binding domain of the SK. Once phosphorylated, the phosphorylated SK serves as the phosphoryl donor to its cognate RR, which autocatalyzes the phosphorylation of a conserved Asp side chain on the surface of its own receiver domain. This phosphotransfer from the SK to the RR requires divalent metal ions and Mg^{2+} is thought to be the prevalent cation used *in vivo*. Phosphorylation of the conserved Asp residue leads to a conformational change in the RR, allowing it to alter the molecular interaction(s) by which the response is achieved. The final phosphotransfer occurs from the phosphorylated Asp

to water via hydrolysis, bringing the RR back to its unphosphorylated state[reviewed in (127)].

The half-life of phosphorylated RRs vary drastically, but typically ranges from a few second to hours (51). However, there are some exceptions. For example, the yeast response regulator SSK1 has a half-life of approximately 2 days (59). Several factors contribute to this short half-life: (i) phospho-Asp possesses an intrinsic instability which favors its unphosphorylated state (1); (ii) many RRs possess autophosphatase activity, which helps increase the rate of dephosphorylation (51); and (iii) Many SKs also contain phospho-Asp phosphatase activity, which is also thought to contribute to the dephosphorylation rate of their cognate RRs [reviewed in (43, 74)]. This phospho-Asp phosphatase activity has been demonstrated both *in vivo* and *in vitro* for many SK. However, in some cases, it has been proposed that the phosphatase rate is too slow for it to be relevant *in vivo* (66).

THE CpxAR TWO-COMPONENT SIGNAL PATHWAY

The *E. coli* K-12 genome encodes 30 SKs and 32 RRs (93). The RR CpxR and its cognate SK CpxA constitute the 2CST pathway CpxAR (**Fig. 2**). This signal pathway is highly conserved in the family Enterobacteriaceae and can be found in some other Proteobacteria. In *E. coli*, the CpxAR pathway regulates transcription of at least 50 genes (29, 102), including genes that control part of the envelope stress response system, pilus assembly, type III secretion, motility and chemotaxis, adherence, and biofilm development. Furthermore, the CpxAR pathway is required for in-

vasion of host cells in diverse pathogenic bacteria, including *E. coli* (enterohemorrhagic *E. coli* and uropathogenic *E. coli*), *Salmonella enterica* serovar Typhimurium, *S. enterica* serovar Typhi, *Shigella sonnei*, *Yersinia enterocolitica*, *Heamophilus ducreyi* and *Legionella pneumophila* [for reviews, see references (31, 32, 106, 109, 110, 115, 122)]. A recent study demonstrated that *Xenorhabdus nematophilus* requires the Cpx pathway both to colonize its nematode host (*Steinernema carpocapsae*) and to kill larvae of the tobacco hornworm (*Manduca sexta*). In *L. pneumophila*, the pathway regulates the type IV bundle-forming pilus (BFP) and some components of type IV secretion, respectively [reviewed in (137)]. BFP assembly is also affected by the CpxAR system in enteropathogenic *E. coli* (EPEC). In this organism, a deletion of CpxR decreased BFP and affected its ability to adhere to epithelial cells (97).

The CpxAR is an autogenously regulated 2CST pathway. CpxA, a SK with autokinase, phosphotransfer, and phospho-CpxR phosphatase activities (36, 111), is located in the cytoplasmic membrane, where it senses diverse signals, including alkaline pH, altered membrane lipid composition, interaction with hydrophobic surfaces, and misfolded pilin subunits, as well as exposure to copper, detergents, and EDTA. In response to these different stimuli, CpxA autophosphorylates and serves as a donor of phosphoryl groups to CpxR, the cognate RR. By analogy to the RR OmpR, CpxR phosphorylation is thought to occur on the conserved Asp-51 residue on its receiver domain.

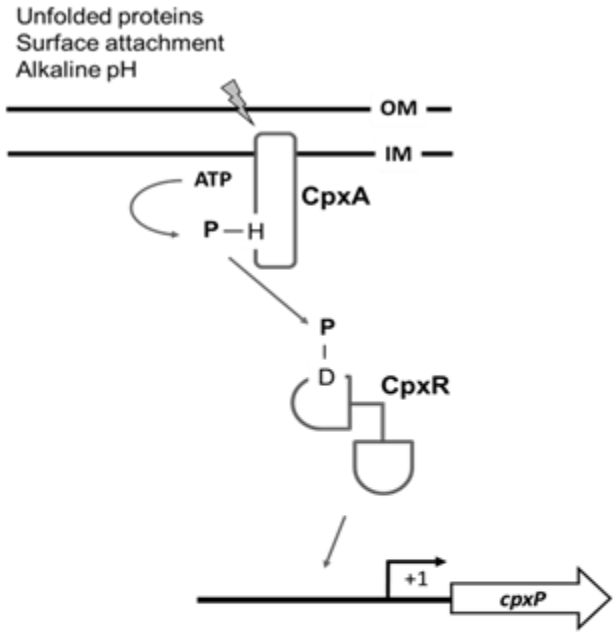


Fig 2. The two-component CpxAR
Activation by the cytoplasmic membrane sensor kinase CpxA. Upon activation by diverse extracytoplasmic signals, CpxA autophosphorylates on a conserved histidine residue, using ATP as its phosphoryl donor. Phospho-CpxA then acts as a phosphoryl donor to the response regulator CpxR, which autophosphorylates on the conserved aspartate residue (D51). By homology to OmpR, phosphorylation of CpxR is predicted to expose its DNA-binding domain, which promotes binding to its target genes (2,6).

The CpxR pathway includes at least two more upstream components. The first additional component, CpxP, was identified as an alkaline-induced member of the Cpx regulon (23). This periplasmic chaperone binds to the periplasmic domain of CpxA and inhibits its autokinase activity (36, 107, 108). It appears that CpxP binds to specific features of certain misfolded proteins, making them available to the ATP-independent periplasmic protease DegP. Since CpxP is degraded along with its cargo, misfolded proteins in the periplasm enhance the probability that CpxA will function as a kinase and initiate the phosphotransfers that will end with activation of the Cpx regulon including CpxP (11, 30, 54, 77). The second component, the outer membrane lipoprotein NlpE, activates CpxA when it is overexpressed (120), and it is required for activation of CpxA in response to adherence to hydrophobic surfaces (98). Since adhesion requires NlpE, but not CpxP, and since stresses that require CpxP do not require NlpE (23, 30, 98), CpxA must integrate information from at least these two distinct stress response pathways (30, 107). Furthermore, it is likely that additional upstream components exist (107). For example, CpxA and CpxR-dependent resistance to copper exposure requires neither CpxP nor NlpE (151).

In the absence of its extracytoplasmic stimuli, CpxA is reported to function as a net phosphatase, removing phosphoryl groups from phospho-CpxR (85, 111, 149). Like many RRs, purified CpxR can accept a phosphoryl group from AcP (101, 111). AcP, the high-energy intermediate of the phosphotransacetylase (Pta)-acetate kinase (AckA) pathway (**Fig. 3**), has a larger $\Delta G°$ of hydrolysis (-43.3 kJ/mol) than ATP (-30.5 kJ/mol in complex with Mg^{2+}). This large amount of energy stored by AcP is

crucial for its role as a global signaling molecule, as this energy is capable of inducing the conformational changes required for activation of RRs (60, 129). The phospho-Asp phosphatase activity of CpxA, therefore, could function to prevent inappropriate AcP-dependent activation of CpxR. In this dissertation I will address these issues by testing if CpxA possess phosphatase like-activity *in vivo*, and whether AcP function as a phosphoryl donor to CpxR *in vivo.*

PTA-ACKA PATHWAY AND ACETYLPHOSPHATE

Reversible *in vivo*, the Pta-AckA pathway interconverts coenzyme A (CoA), ATP, and acetate with acetyl-CoA (AcCoA), ADP, and inorganic phosphate (Pi). This reversibility permits both AcCoA synthesis (acetate activation) and acetate evolution (acetogenesis) (**Fig. 3**). During acetogenesis, Pta synthesizes AcP and CoA from AcCoA and P_i, while AckA generates ATP from AcP and ADP.

Simultaneously, AckA produces acetate, which cells freely excrete into the environment. Thus, the steady-state concentration of AcP depends on the rate of its formation catalyzed by Pta and the rate of its degradation catalyzed by AckA [reviewed in (145)]. Acetogenesis has several key functions. It recycles CoA, facilitating glycolytic flux and hence rapid growth in the presence of an excess of a preferred carbon source (e.g., glucose or pyruvate) [reviewed in (145)]. This function also can reinitiate stalled tricarboxylic acid (TCA) cycle function, providing CoA to convert α-ketoglutarate to succinyl-CoA (35). Critically, acetogenesis provides the majority of ATP in the absence of robust TCA cycle activity (145).

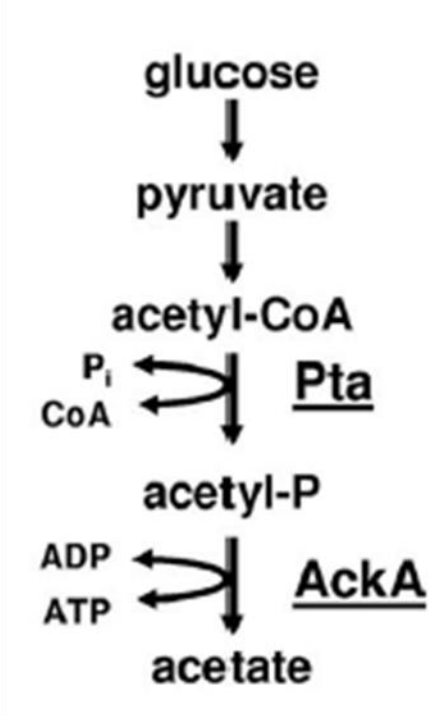


Fig. 3. Schematic diagram of the Pta-AckA pathway that converts acetyl-CoA to acetate via an AcP intermediate.

However, the conversion of AcCoA to acetate and ATP often does not go to completion; *E. coli* cells can and do maintain a significant pool of AcP (72, 90, 104). This pool serves the cell in two distinct and equally important ways. First, AcP serves as a storage molecule for carbon (C), phosphate (OPO_3), and energy in the form of its high-energy $COOPO_3$ bond (92). Second, AcP can serve as a signaling molecule [reviewed in (146)].

In the early 1990s, AcP was proposed to act as a global signal by donating its phosphoryl group to a subset of RRs (91, 141). Intracellular AcP concentration varies in response to the environment. AcP concentrations can be as high as 4 to 5 mM in wild-type (WT) cells and 15 to 20 mM in *ackA* mutants (72). These concentrations are sufficient to induce *in vitro* phosphorylation of some RRs (84).

Much evidence now exists to support the hypothesis that AcP-dependent phosphorylation of a subset of RRs contributes to their function *in vivo* [reviewed in (146)]. For example, the RR RcsB can be induced to activate capsule biosynthesis and repress flagellar biogenesis in an AcP-dependent manner (38). Genetic evidence in support of this proposition was obtained using epistasis analysis. By combining mutations in the Pta-AckA pathway with mutations in the Rcs 2CST pathway and then comparing the phenotypes of the resultant double mutants to those of the parental single mutants, Fredericks et al. were able to conclude that AcP acts via RcsB to activate genes for encapsulation and to repress genes for flagellation (38). This well-characterized DNA-binding RR controls about 5% of the *E. coli* genome [reviewed in (87, 103)] and as much as 20% of the *S. enterica* genome (140), including

many genes involved in virulence. Thus, AcP influences expression of multiple genes through a known global regulator.

While many behaviors associated with disruption of the Pta-AckA pathway correlate with AcP concentrations, some do not [reviewed in (146)]. If a behavior strictly depends on AcP to donate its phosphoryl group to a response regulator, then an *ackA* mutant, which accumulates AcP (72), should elicit a behavior opposite to that exhibited by a *pta* mutant, which cannot synthesize AcP (72). However, that is not always the case. This lack of correlation could be due to a lack of involvement of AcP, or perhaps because disruption of the Pta-AckA pathway can have more pleiotropic effects on central metabolism. For example, disruption of the Pta-AckA pathway is likely to affect the flux of acetyl groups through central metabolism [reviewed in (144)].

ACETYL-COENZYME A

AcCoA plays a very important role in cellular metabolism (**Fig. 4**) . It contributes to fatty acid biosynthesis and serves as the carbon donor to the TCA cycle by donating its acyl group to oxaloacetate. This transfer of acetyl groups recycles CoA [reviewed in (144)], the major acyl group carrier in living systems (78). *E. coli* produces limiting amounts of CoA, and growth arrest initiates once the CoA concentration falls below 5 pmol/10^8 cells (58). Therefore, cells must recycle CoA to maintain proper glycolytic flux and therefore growth.

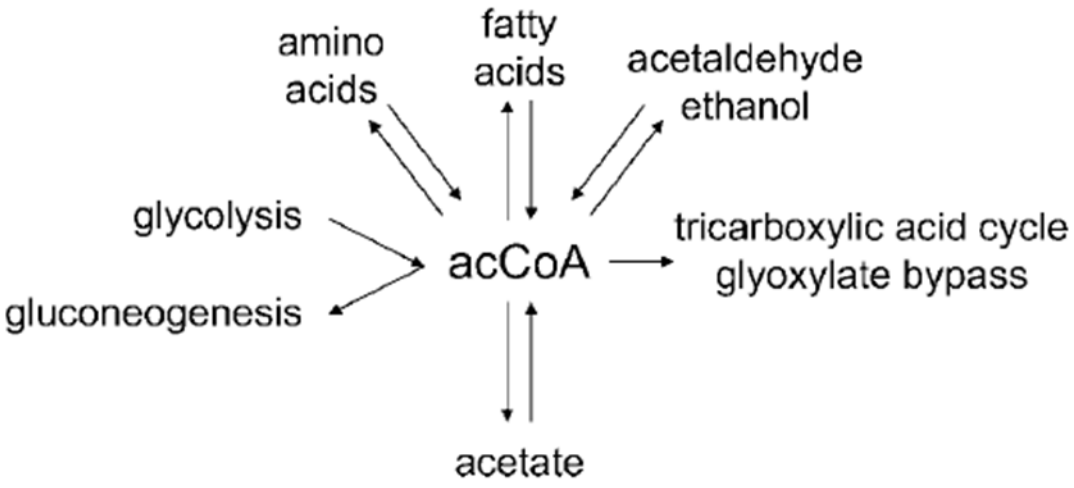


Fig. 4 Acetyl-CoA (acCoA) sits at the crossroads of central metabolism.

Similar to AcP, AcCoA concentration has been shown to fluctuate and rapidly increase when *E. coli* cells are exposed to some carbon sources like glucose (135). This increase in AcCoA concentration has the potential to put stress on the limited CoA pool, potentially leading to metabolic imbalance. When combined with disruption of the Pta-AckA pathway, this imbalance could drastically affect cellular physiology.

ACETYLATION

Protein acetylation is typically thought of as the transfer of an acetyl group from an acetyl donor, e.g. AcCoA, to a protein. This transfer can occur either at the amino terminus of a protein (Nα) or to the ε-amino (Nε) group of a lysine residue (**Fig. 5**). This transfer is often mediated by an acetyltransferase that can establish the proper catalytic microenvironment. The acetyltransferase acts as a scaffold, orienting the acetyl group of AcCoA and the amino group of the acceptor protein into close proximity. The enzyme then promotes deprotonation of the amino group, creating a neutral amino group that serves as a nucleophile that attacks the carbonyl carbon of AcCoA, acetylating the protein and releasing a free CoA molecule (33).

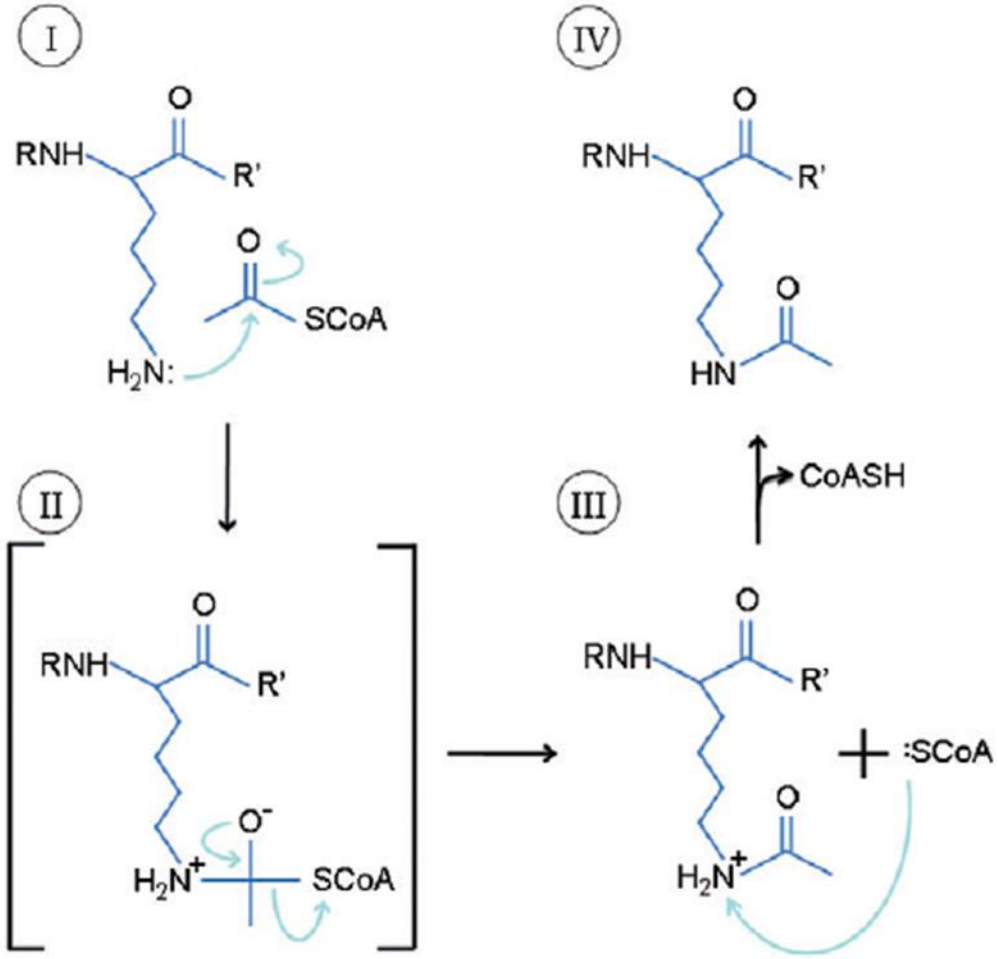


Fig. 5. Electron transfer during direct Ne-acetylation from acetyl-CoA. At neutral pH, the e-amino group is positively charged. This residue is deprotonated by a base (not shown in figure). (I) The lysine e-amino group can now act as a nucleophile to attack the electrophilic carbonyl carbon of acetyl-CoA (II) forming a tetrahedral intermediate. The electron dense oxygen expels the thiolate group (-SCoA). (III) Deprotonation of the amino group (IV) results in an acetylated lysine side chain and CoASH. Hu et al .

Nα-acetylation is considered to be an irreversible modification of either the deformylated N-terminal methionine or, following cleavage of the N-terminal methionine, the newly exposed amino acid. In eukaryotes, Nα-acetylation of proteins is extremely common (present on more than 80% of mammalian proteins) and is primarily cotranslational. In bacteria, Nα-acetylation is proposed to be less common, occurring post-translationally on ribosomal proteins [reviewed in (53)].

In contrast to Nα-acetylation, Nε-acetylation is both dynamic and reversible. This property permits Nε-acetylation to serve in as regulatory mechanism. The impact of Nε-acetylation varies according to the context. Nε-acetylation can change the size, charge and/or conformation of the protein. These changes can alter DNA binding affinity, protein stability, protein–protein interactions, protein localization and protein function. Nε-acetylation is also known to influence other post-translational modifications [reviewed in (46, 152)], a process known as PTM crosstalk. In general, evidence of PTM crosstalk is more prevalent in eukaryotic organisms. For example, gene expression can be regulated at the level of chromatin structure. Numerous residues within the histone tails and several residues within the histone globular domains can be modified in a variety of ways, including acetylation, phosphorylation, ubiquitination, and methylation[reviewed in (76)]. The combination of these modifications, depending to the context, can act to facilitate or repress transcription. In some cases the modification of one residue can alter the ability of a second residue to be implemented by its modifying enzyme(s). For example, phosphorylation of

serine 10 on histone H3 stimulates the ability of Gcn5 to acetylate histone H3 at lysine 14 (H3K14) (19, 86). A second example of PTM crosstalk in eukaryotes can be seen with CTD of eukaryotic RNA polymerase II which has been shown to be both phosphorylated and glycosylated. The pattern or glyscosylation and/or phosphorylation affect the ability of some proteins to interact with RNAP II. Although both glycosylation and phosphorylation can occur on RNAP II CTD, phosphorylation seems to prevent glycosylation [reviewed in (34)]. The evidence is less extensive that PTM cross-talk occurs in bacteria; however, multiple proteins have been shown to be modified by more than one PTM [reviewed in (121)]. For example, the RRs RcsB and CheY have been shown to be both phosphorylated and acetylated and evidence exists to support the hypothesis that the function of these proteins is affected by both PTMs (82, 132). While it remains to be determined whether either PTM can affect the other, some evidence exists that *in vitro* phosphorylation of RcsB might be affected by its acetylation (Hu, L. I. and Wolfe, A. J. unpublished)

Acetylation of non-lysine residues also has been shown to occur. In bacteria, Ser/Thr acetylation is known to have a role in virulence. For example, *Yersinia pestis* blocks host defense pathways in the inflammatory response by acetylating serine and threonine residues that are essential in MAPK/NF-κB signaling pathways (94). *Salmonella enterica* utilizes a similar mechanism during host invasion (63).

Unless otherwise mentioned, for the remainder of this section, I will focus of the reversible Nε-Acetylation.

While in eukaryotic cells, protein acetylation has been extensively studied, in bacteria and archaea this has not been the case. Until recently, our knowledge of protein acetylation in bacterial cells was limited to the acetylation of acetyl-CoA synthetase (an enzyme that converts acetate to AcCoA) and CheY (a signal protein central to chemotaxis) [reviewed in (53, 131)]. However, recent global proteomics studies have provided evidence that protein acetylation is widely prevalent in *E. coli* (155, 157), *S. enterica* (139) and more recently *B. subtilis* (68). These studies indicate that Nε-lysine acetylation might be as prevalent in bacteria as in eukaryotes.

In parallel with what has been reported for eukaryotic cells, protein acetylation in bacteria is thought to be regulated and reversible [reviewed in (53, 130)]. Protein acetylation is thought to be mediated by proteins with lysine acetyltransferase activity (KAT), while deacetylation is thought to be mediated by proteins with lysine deacetylases (KDACs)

Up to five groups of proteins with KAT activity have been identified: (i) the Gcn5-related acetyltransferase (GNAT) family, (ii) the MYST family, (iii) the CBP/p300 co-activators, (iv) the SRC family of co-activators and (v) the TAFII group of transcription factors [reviewed in (88, 126)].

Of the five groups of proteins with acetyltransferase activity, the GNATs are the most widely distributed, with over 10,000 members identified across all three domains of life [reviewed in (136)]. Importantly for bacteriologists, GNATs are ubiquitous within the bacterial domain and are capable of carrying out a diverse range of functions. Some GNATs, such as protein acetyltransferase (Pat) from *S. en-*

terica, catalyse Nε-acetylation (124), while other GNATs are reported to perform Nα-acetylation; for example RimI, RimL and RimJ of *E. coli* (128, 154).

Not all GNATs catalyse protein acetylation. Indeed, the first characterized members of the GNAT family, the aminoglycoside *N*-acetyltransferases, were discovered as bacterial enzymes that could acetylate and thus inactivate antibiotics [reviewed in (27, 118)].

In addition to this acetyltransferase mediated acetylation of proteins, autoacetylation, an intramolecular covalent modification, has also been reported for several eukaryotic KATs [Tip60 (138) and p300 (133) for example] and for the *E. coli* proteins acetyl-CoA synthetase (ACS) and CheY (4). Unpublished evidence from the Wolfe laboratory adds the RR RcsB and the central metabolism protein LpdA to the list of proteins that appear to autoacetylated (Linda I. Hu, Bui Khanh Chi, Misty L. Kuhn, Ekaterina V. Filippova, Arti J. Walker-Peddakotla, Katrin Bäsell, Dörte Becher, Wayne F. Anderson, Haike Antelmann, and Alan J. Wolfe unpublished data).

Two major families of KDACs have been identified. These two families have been grouped into four classes (49): the zinc-dependent Rpd3/Hda1 family (classes I, II and IV) (153) and the NAD^+-dependent sirtuin family (class III) (8). For each KDAC class, putative bacterial homologues have been identified [reviewed in (52)], but only a few have been shown to serve as protein deacetylases and very few of their protein substrates have been identified (44, 45, 52, 79, 123, 139).

RNA POLYMERASE AND TRANSCRIPTION INITIATION

The *E. coli* genome encodes approximately 4500 open reading frames (ORF). However, the number of RNAP subunits available for transcription is less than half of that, about 2000 units per cell [reviewed in (55)]. Thus, a large effort is invested in the decision as to which promoter(s) will be engaged by RNAP, and thus which genes will be transcribed. Because RNAP is a limited resource, transcription initiation is the major regulatory mechanism used to regulate genomic expression in bacteria.

RNAP is a large multi-subunit complex. Its core (E) is composed of 5 highly conserved subunits, $\alpha_2\beta\beta'\omega$, with homologues found in all domains of life. Although this core enzymatic complex is capable of synthesizing RNA polymers, its ability to bind DNA requires interaction with one of the seven σ factors encoded by the *E. coli* genome. The complex formed by E and one of the σ factors is called RNAP holoenzyme (Eσ). In *E. coli,* σ^{70}, encoded by *rpoD*, is the most abundant σ factor and it is required for transcription of most ORFs [reviewed in (50, 56)].

Although σ^{70} is the most abundant σ factor in *E. coli*, the cellular concentrations of the σ factors are very dynamic. It varies with different growth phases and environmental conditions (55, 57). This change in steady state levels of σ factors will, consequently, change the probability that E will interact with a particular type of σ factor. Since different promoters, or groups of promoters, possess binding elements that favor the binding of different σ factors, the concentration of the sigma

factors will dictate the genes that can be transcribed at any given time [reviewed in (50, 56, 114)].

After interaction with one of the σ factors, the Eσ complex is now capable of interacting with a promoter region. The complex DNA-Eσ is termed RNAP closed complex (RPc). After the initial promoter interaction, the next step in transcription initiation leads to melting of the double stranded DNA and the formation of a transcription bubble that begins at a nucleotide hexamer known as the -10 element, because it is located approximately 10 nucleotides upstream of the transcription start site, which is also included in the transcription bubble. This complex is known as the open complex (RPo). With the exception of $E\sigma^{54}$, the energy required to drive the melting of the double stranded DNA comes from large conformational changes in Eσ and DNA and not from ATP hydrolysis or the hydrolysis of any other energy filled metabolite. Once the open complex is formed, NTP incorporation drives the transcription reaction forward. This step is known at the transcription initiation complex (TIC). However, at most promoters, RNAP synthesizes short, abortive products before transitioning to the elongation complex (TEC). During this time, the leading edge of σ and the active site of the enzyme move forward, but the contacts between the trailing edge of RNAP and the nucleotide hexamer located approximately 35 nucleotides upstream of the transcription initiation site(–35 hexamer) remain intact. Transcription elongation occurs once Eσ breaks its contact with the promoter region and progresses downstream. Eventually, the interaction between E and the σ factor also breaks and σ releases to interact with another E (50). Although a large

amount of molecular information is known about the $E\sigma^{70}$ closed and open complexes, not much is known about the intermediate stages [reviewed in (50, 56, 114, 147)].

Availability and interaction with the different σ factors is only one of the many factors determining transcription initiation. Further selectivity is achieved by interaction of Eσ with one of the approximately 300 transcription factors in *E. coli*. While most of these transcription factors contain DNA-binding elements, approximately 10% of them interact directly with RNAP, regulating transcription elongation and termination (57). Similarly to σ factors, the concentration of these transcription factors is affected by growth phases and environmental conditions, as well as nutritional status. In general, the vast majority of these transcription factors are present in very small concentrations, usually less than 100 molecules per cell. Most transcription factors possess very weak affinity for RNAP, which in some cases can be altered via a covalent posttranslational modification or a non-covalent interaction with a ligand. It is important to mention that although some of these modifications can increase the affinity between transcription factors and RNAP, the overall affinity between them must remain relatively weak to allow for a quick turnover of transcription factor that interacts with RNAP. This relatively weak interaction is also required to facilitate promoter clearance, as a complex that is too stable will have difficulty breaking free from the many interactions formed at the promoter level, and will be less likely to progress into transcription elongation [reviewed in (50, 56)].

In addition to binding elements for σ factors, and in some cases binding sites for some transcription factors, some promoters may also contain a stretch of adenosines (A) and thymidines (T) upstream of the σ factor interaction site. This AT-rich stretch, termed the UP-element, can serve as an additional stability point as the carboxy-terminal domain of the α subunit of RNAP (αCTD) can make direct contact to this region (48, 113).

The α subunit is composed of two independently folded domains tethered by a flexible linker region of approximately 15 amino acids (9). Dimerization of the amino-terminal domain (αNTD) is the nucleating step for E assembly (156), while the CTD directly interacts with DNA, in and across a minor groove, via a helix-hairpin-helix motif (7, 48, 112).

The UP-element is widely distributed in bacteria, plasmids and phages. Genetic and biochemical analysis of the interaction between αCTD and the UP-element in the *rrnB* promoter has identified a crucial patch of amino acids (Arg-265, Asn-294, Gly-296, Lys-298 and Ser-299) on the αCTD, known as the 265 determinant, that is required for DNA interaction (7, 112). This information has been confirmed, at the atomic level, by X-ray structure analysis of the interaction between αCTD and the UP-element on the *lac* promoter (7). In addition to directly interacting with DNA, the 265 determinant has been shown to be directly involved in a protein-protein interaction between αCTD and the transcription elongation protein NusA (116).

CHAPTER TWO

MATERIALS AND METHODS

BACTERIAL STRAINS, BACTERIOPHAGE AND PLASMIDS

All bacterial strains used in this study are listed in Table 1. Derivatives were constructed by generalized transduction with P1kc (for details, see *Generalized P1 transduction*). The transcriptional fusion ϕ(*PcpxP*'-*lacZ*) carried by λ*PcpxP* was a generous gift from Thomas Silhavy (Princeton University, Princeton, NJ). All plasmids used in this study are listed on Table 1. Derivatives were constructed either via cloning or by site-directed mutagenesis (for details, see *Plasmid Construction* and *Site-Directed Mutagenesis*).

CULTURE CONDITIONS

For strain construction, cells were grown in Luria Broth (LB) containing 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, and 0.5% (wt/vol) potassium chloride; LB plates also contained 1.5% agar. For promoter activity assays, cells were grown in tryptone broth containing 1% (wt/vol) tryptone and 0.5% (wt/vol) potassium chloride. Potassium chloride was used instead of sodium chloride because sodium has a negative effect on the survivability of WT cells at alkaline pH (64). For some experiments, the medium (liquid or solid) was buffered with base and acid to

a final concentration of 100 mM at ratios required to achieve the desired pH. No KCl was added to TB when a potassium-based buffer was used to adjust the pH. Cell growth was monitored spectrophotometrically (DU640; Beckman Instruments, Fullerton, CA) by determining the optical density at 600 nm (OD600).

Antibiotics were prepared as a stock solution 1000 times the working concentration. Working concentrations were as follows: ampicillin, 100 μg/mL; kanamycin, 40 μg/mL; spectinomycin 100 μg/mL; tetracycline, 15 μg/mL and chloramphenicol, 25 μg/mL. Stock solutions of ampicillin, kanamycin and spectinomycin were dissolved in water and filter sterilized. Stock solutions of tetracycline and chloramphenicol were dissolved in 50% and 100% ethanol, respectively, and filter sterilized. All antibiotic stocks were stored at -20°C. When necessary, isopropyl β-D-1-thiogalatopyranoside (IPTG) was added to the media at the indicated concentration to induce gene expression from a plasmid vector. Nile red (Sigma Aldrich), at a final concentration of 25 μg/ml, was added to LB plates to detect *in vivo* PHB accumulation. PHB detection using Nile red will be explained later.

GENERALIZED P1 TRANSDUCTION

A single colony was inoculated in TB and grown overnight at 37°C aerobically with agitation at 250 RPMs. In the morning, 50 μL of overnight culture were added to 5 mL of TB for transduction (TBT) (0.2% [wt/vol] glucose from a 20% [wt/vol] stock solution, 10 mM $CaCl_2$, 10 mM $MgSO_4$, and 0.4 mM $FeCl_3$ in TB). The overnight culture was grown in TBT until it reached an OD600 of approximately 0.5. One mL of

cells was infected with 10-100 µL of phage lysate and incubated statically at 37°C for 30 minutes. After incubation, 200 µL of 1M sodium citrate (pH 5.5) were added to prevent a second round of phage absorption. The cells were pelleted using a table-top centrifuge at 16000 RCF for 1 minute at room temperature. The supernatant was decanted and the pellet resuspended with 500 µL LB and 200 µL 1M sodium citrate. The cells were then incubated aerobically at 37°C with agitation at 250 RMP for 70 minutes to give them time to develop antibiotic resistance. After the second round of incubation, the cells were pelleted as described before. The supernatant was decanted and the pellet was resuspended with 50 µL 1 M sodium citrate. The entire cell suspension was plated onto LB agar plates with appropriate antibiotics required for the desired selection.

PLASMID CONSTRUCTION

Plasmids pPHB1 and pPHB3. These plasmids were constructed by Shaun R. Brinsmade (UW-Madison). Alleles *phbCAB*$^{+}$ were amplified from *Ralstonia solanacearum* strain GMI1000 (a kind gift from Caitilyn Alen, UW-Madison) using forward primer 5'—ATC GAA CCC GGG ATG GCA TCG CGT CAC AA—3' and reverse primer 5'—AGC ATG AGT GCC CCG GGT CAG CCC ATG T—3'. Bases underlined indicate the *Sma*I restriction site engineered into each primer. The resulting ~4-kb fragment was A-tailed and gel-purified using the QIAquick® gel extraction kit (Qiagen). This product was ligated into the multiple cloning site of plasmid pGEM-T-Easy (Promega). The resulting plasmid was ~7-kb. From this plasmid, named pPHB1, the *Rs phbCAB*$^{+}$

genes were cut out using *Sma*I. The resulting ~4-kb fragment was gel-extracted and ligated into plasmid pTAC-85 (89) that had been cut with *Nco*I and the resulting single-stranded DNA overhangs blunted with mung bean nuclease and dephosphorylated using shrimp alkaline phosphatase. The resulting plasmid was ~9.1-kb and was named pPHB3.

Plasmid pBPL001. The WT *cpxR* allele was amplified from pCA24n-CpxR using primers cpxRFNdeI (5'-GGTATTTAACATATGAATAAATCCTG-3') and cpxRRHindIII (5'-GGTATTTAACATATGAATAAAATCCTG-3'). The resulting amplicon was ligated into pJET1.2, using the CloneJET™ PCR Cloning Kit (Fermentas). The ligated product was cut out of pJET1.2 with *Nde*I and *Hin*dIII, gel-extracted, ligated into pET28 downstream of the IPTG-inducible promoter, and fused to a C-terminal His_6 tag. The resulting plasmid was named pBPL001.

Plasmid p770-cpxP. This plasmid is a derivative of *p770* (a generous gift from Rick Gourse at the University of Wisconsin-Madison). It is an ampicillin-resistant ColE1-based plasmid that has the tandem *rrnB* operon terminators T1 and T2 located downstream of the insertion sites for the promoter. The *cpxP* promoter region was amplified from strain BW25113 with the following primer pair: cpxP'FEcoRI (5'-AATAGGGAATTCAGTTCTCGGTCATC-3') to insert the *Eco*RI site upstream of the promoter region and cpxP'RHinDIII (5'-GCAGCGAAGCTTAATGAACTGACTG-3') to insert the *HinD*III site downstream. The resulting amplicon was ligated into pJET1.2,

using the CloneJET™ PCR Cloning Kit (Fermentas) following kit instructions. The ligated *cpxP* insert was sequenced, digested out of pJET1.2 with *Eco*RI and *Hin*dIII, and gel-extracted. The gel-extracted fragment was cloned into previously digested *p770* via overnight ligation at room temperature. Ligated plasmids were transformed into *E. coli* DH5α cells. Plasmids were recovered from transformants and restriction digested to screen for the presence of the *cpxP* promoter. The presence of the promoter was confirmed via sequencing analysis. The plasmid containing the correct insert was purified, stored at -20°C and named *p770-cpxP*.

SITE DIRECTED MUTAGENESIS

Site directed mutagenesis of plasmids *pBPL001, pIA900* and *pREII* was conducted using Agilent Technologies's QuickChange Site-Directed Mutagenesis Kits, according to manufacturer's instructions. The mutations were confirmed by sequence analysis of the purified mutagenized plasmids.

TRANSFORMATION

Two distinct transformation methods were used: TSS and electroporation. TSS – 1 ml of overnight cell culture was pelleted at room temperature for 1 minute at 16000 RCF using a table-top centrifuge. The pellet was resuspended with 100 µl TSS [10% (wt/vol) poly(ethylene glycol), 5% (vol/vol) DMSO and 50 mM MgSO4 or MgCl2 in LB]. After resuspension, 50 ng of plasmid DNA was added to the resuspended cells, the mixture was incubated on ice for 30 minutes. After ice incubation,

0.9 mL of LB was added, and the cells were incubated for 1 hour at 37°C with aeration. After 37°C incubation, the cells were pelleted one more time as described above, the supernatant was decanted, and the cells were resuspended in 250 µL LB. 25-100 µl of the resuspended cells were used for plating on an LB agar plate containing the appropriate antibiotic for selection.

Electroporation - For electroporation, the recipient cells were made competent by inoculating 30 mL of LB in a 250 mL baffled flask with 500 µL of overnight culture. The culture was incubated with aeration rotating at 250 RPM at 37°C until OD600 reached 0.4-0.6. The culture was split into two Oakridge tubes and spun down at 10000 RCF for 10 minutes at 4°C. After pelleting the cells, the supernatant was aspirated, and the cell pellet was resuspended with 1 mL of ice-cold sterile water. After resuspension, an additional 9 mL of ice cold water was added to the tube, bringing the total volume to 10 mL and the cells were spun one more time at 10000 RCF for 10 minutes at 4°C. At the end of the second spin, the supernatant was gently decanted immediately, as the pellet is not very compact and stable at this point. The pellet then was resuspended one more time with 1 mL of ice cold water, transferred to a 1.5 mL eppendorf tube, and spun down at 16000 RCF for 1 minute, using a table top centrifuge at room temperature. The supernatant then was aspirated one more time, the pellet was resuspended with 200 µl of water, divided into 50 µl aliquots and stored at -80°C for future use.

Transformation began by mixing 50 ng of purified plasmid DNA with 50 µL of electro-competent cells. The DNA-cell mixture was then transferred to a 1 mm chilled electroporation cuvette and electroporated at 1.8 KV. After electroporation, the cells were immediately rescued by the addition of 1 mL of LB, transferred to a small test tube, and incubated at 37°C, rotating at 250 RPM for 1 hour. After incubation, 20-100 µL of cells were plated onto LB agar plates containing the appropriate concentration of antibiotic required for selection of transformants. Outgrowth was often, but not always, carried out overnight at 37°C.

PROMOTER ACTIVITY ASSAY

To monitor promoter activity from ϕ(*PcpxP*'-*lacZ*), cells were grown aerobically with agitation at 250 rpm at 37°C in TB that was unbuffered or TB that was buffered either at pH 7.0 (TB7) or at pH 8.0 (TB8). At regular intervals, 50 µL aliquots were harvested and added to 50 µL of All-in-One β-galactosidase reagent (Pierce Biochemical). β -galactosidase activity was determined quantitatively with the Y-PER β-galactosidase assay kit (Pierce Biochemical), using a microtiter format. Promoter activity was plotted versus OD600. For some experiments, only the peak activity is shown. Each experiment included three independent measurements and was repeated at least once.

pH SENSITIVITY ASSAY

Cells were grown aerobically with agitation at 250 rpm at 37°C in LB buffered at pH 7.0 (LB7) until either mid-exponential phase or early stationary phase, harvested, serially diluted in LB7, and plated onto LB plates buffered at the specified pH. The plates were incubated overnight at 37°C, the colonies were counted, and the colony diameters were measured.

NILE RED

Nile Red was used to detect the production and accumulation of polyhydroxybutyrate (PHB) on LB agar plates by *E. coli* cells carrying plasmid *pPHB3*. A single colony was streaked onto LB agar plate containing Nile Red at a final concentration of 0.5 μM/ml, 50 μM IPTG to induce PHB expression and 25 μg/ml ampicillin to force the cells to maintain the plasmid. The plates were incubated overnight at 37°C. After incubation, PHB accumulation was detected by exposing the plates to ultraviolet light (λ=312 nm).

TRANSMISSION ELECTRON MICROGRAPH

Transmission electron micrography was used to detect the production and accumulation of polyhydroxybutyrate (PHB) granules in the cytoplasm of *E. coli* cells. A single colony of strain PAD 282 carrying plasmid *pPHB3* was incubated overnight in 5 mL of TB7 containing 100 μg/mL ampicillin. 2 mL of the overnight culture was collected and the cells were pelleted using a table-top centrifuge at 16000 RCF for 1

minute at room temperature. The cell pellet was resuspended with 1 mL of fixing solution (1.2% glutaraldehyde, 0.45 M cacodylate buffer pH 7.3 and 0.1% ruthenium red) and incubated overnight at room temperature. After overnight incubation, the cells were pelleted at room temperature using a table-top centrifuge at 16000 RCF for 3 minutes, the eppendorf tube was rotated 180° and spun again for 1 min at the same RCF. After the final centrifugation, the supernatant was decanted and the cell pellet was washed for 10 minutes twice with washing buffer (0.45 M cacodylate buffer pH 7.3 and 0.1% ruthenium red). After the second wash, the cell pellet was incubated for 1 hour at room temperature in washing buffer containing 4% osmium tetroxide, after which they were washed twice again for 10 minutes with washing buffer. The cells were then dehydrated by a series of 15-minute ethanol washes at room temperature with increasing concentration of ethanol: 25%, 50%, 75%, 90% and four 15-minute washes with 100% ethanol, followed by two 10-min washes with propylene oxide at room temperature. At the end of the second propylene oxide wash, the pellet was incubated overnight with a 1:1 mixture of resin and propylene oxide, followed by a 7-8 hour incubation with a 3:1 mixture of resin and propylene oxide, ending with an overnight incubation in 100% resin. After the last resin incubation, the samples were placed in molds, filled with fresh resin and placed in the oven overnight, after which the samples were ready to be processed and visualized.

WESTERN IMMUNOBLOT ANALYSIS

For Western Immunoblot analysis, 1.5 ml of cell culture was used. The cells were pelleted by centrifugation. Cell lysis was accomplished by one of the following methods: sonication, resuspension in 2x SDS buffer (120 mM Tris-Cl [pH 6.8], 4% SDS, 0.2% bromophenol blue, 20% glycerol and 10% 2-mercaptoethanol), or Bug-buster™ (according to manufacturer's instructions). The protein samples were re-solved by electrophoresis on a SDS-PAGE 12% polyacrylamide gel, transferred to PVDF or nitrocellulose membrane, and subjected to Western immunoblot analysis.

Protein detection occurred by incubating the membrane with primary anti-body in 5% milk solution overnight at 4°C with shaking. Secondary goat anti-rabbit (Cell Signaling Technology®) immunoglobulin G antibody at a 1:1000 dilution, or goat anti-mouse (Millipore) immunoglobulin G antibody at 1:5000 dilution both conjugated to horseradish peroxidase were used for 1 hour at room temperature with shaking. For visualization, enhanced chemiluminescence Western immunoblot-ting reagents (Cell Signaling Technology®) were used, according to the manufactur-er's instructions.

When necessary, membrane stripping was performed with OneMinute® Western Blot Stripping Buffer (GM Biosciences), according to the manufacturer's instructions.

IMMUNOPRECIPITATION

For immunoprecipitation, 50 mL buffered TB cultures were collected after 7.5 hours of incubation at 37°C, pelleted by centrifugation, resuspended in 5 mL TE buffer and lysed by sonication. 1 mL of lysate was used for immunoprecipitation of RNAP with RNAP β mouse monoclonal antibodies (NeoClone Biotechnology) or immunoprecipitation of CpxR with MBP-CpxR rabbit polyclonal antibody (111) and rotated overnight at 4°C. Protein G-Sepharose® (Sigma) was used to 'pull down' antibodies from cell lysate. The precipitated beads were washed three times with TE buffer and once with wash buffer containing 100 mM NaCl and 50 mM Tris-HCl at pH 7.2. Loading buffer was added directly to the beads and samples were heated to 95°C for 5 minutes. Samples were separated by SDS-PAGE and stained with NOVEX® Colloidal Blue Stain (Invitrogen), according to the manufacturer's instructions.

ORBITRAP-MASS SPECTROMETRY AND PROTEIN IDENTIFICATION

The α, β and β' bands were excised and subjected to tryptic digestion, as described previously (20). Tryptic peptides were separated and measured online by ESI-mass spectrometry using a nanoACQUITY UPLC™ system (Waters, Milford, MA) coupled to an LTQ Orbitrap™ XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA). A trap column (Symmetry® C18, 5 µm, 180µm inner diameter x 20mm, Waters) was used for desalting. Elution was performed onto an analytical column (BEH130 C18, 1,7 µm, 100 µm inner diameter x 100mm, Waters) by a binary gradi-

ent of buffers A (0.1% (v/v) acetic acid) and B (99.9 % (v/v) acetonitrile, 0.1% (v/v) acetic acid) over a period of 80 min with a flow rate of 400 nl/ min. The Orbitrap XL was operated in data-dependent MS/MS mode using the lockmass option for real time recalibration.

Proteins were identified by searching all MS/MS spectra in "dta" format against an *E. coli* database (extracted from the Uniprot-KB database: http://www.uniprot.org/uniprot/?query=Escherichia+coli+K12&sort=score) using **Sorcerer™-SEQUEST®** (Sequest v. 2.7 rev. 11, Thermo Electron including Scaffold_3_00_05, Proteome Software Inc., Portland, OR). The Sequest search was carried out considering the following parameters: a parent ion mass tolerance - 10 ppm, fragment ion mass tolerances of 1.00 Da. Up to two tryptic miscleavages were allowed. Methionine oxidation (+15.99492 Da), cysteine carbamidomethylation (+57.021465 Da) and lysine acetylation (+42.010571 Da) were set as variable modifications. Proteins were identified by at least two peptides applying a stringent SEQUEST filter. Sequest identifications required at least ΔCn scores of greater than 0.10 and XCorr scores of greater than 1.9, 2.2, 3.3 and 3.8 for singly, doubly, triply and quadruply charged peptides. Acetylated peptides that passed these filter criteria were examined manually and accepted only when b^- or y^- ions confirmed the acetylation site.

CpxR EXPRESSION AND PURIFICATION

CpxR expression was carried out in BL21 (DE3) cells transformed with *pBPL001*. An overnight culture of the transformants was used to inoculate 1 L of LB at an initial OD600 of 0.05. The culture was incubated at 37°C with shaking at 250 RPM. Once the OD_{600} reached 0.6, 250 µM IPTG was added to the culture to induce CpxR expression. After three hours of induction, the cell pellet was collected by centrifugation at 10000 RCF for 10 minutes at 4°C and stored at -20°C overnight.

Cell lysis began by resuspending the pellet with 10 mL BugBuster (Novagen®) and 10 µL lysonase™ (Novagen®). The resuspended cell pellet was incubated for 30 minutes at room temperature with gentile shaking. Following this room temperature incubation, 30 mL of pH 8.0 resuspension buffer (50 mM Na_2HPO_4, 1.4 M NaCl, 20 mM imidazole, 0.1% Tween 20, 5% ethanol and 15 mM β-mercaptoethanol) was added to the resuspended pellet. The cell debris was pelleted by centrifugation at 15000 RCF, at 4°C for 30 minutes. The supernatant was loaded onto a 500 µL TALON® Metal Affinity Resin (Clontech) column, washed with 10 mL wash buffer (50 mM Na_2HPO_4, 0.3 M NaCl, 30 mM imidazole, 0.1% Tween 20 and 0.5% ethanol) buffered at pH 8.0. CpxR elution was performed in a stepwise manner by flowing 500 µL of wash buffer with increasing imidazole concentration from 35 mM to 85 mM in 5 mM increments. Fractions 6-11 were collected, combined and dialysed overnight at 4°C into storage buffer (10 mM Tris-HCl pH 8.0, 100 mM KCl, 50% glycerol, 10 mM $MgCl_2$, 0.1 mM EDTA and 1 mM DTT). The dialysed protein was divided into aliquots, which were stored at -80°C for future use.

IN VITRO PHOSPHORYLATION

In vitro phosphorylation of CpxR was carried out by incubating lithium potassium AcP (Sigma Aldrich) with purified His-tagged CpxR at 30°C for 15 minutes in buffer containing 40 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 40 mM KCl and 1 mM DTT. The reaction was quenched by the addition of SDS loading buffer.

DETECTION OF PHOSPHORYLATED CpxR

Whether from cell lysates or from *in vitro* phosphorylation, phosphorylated CpxR was detected by first separating phosphorylated from nonphosphorylated CpxR using Zinc (II) Phos-Tag™ SDS- PAGE [10% acrylamide (29:1), 350 mM Tris pH 6.8, 0.1% SDS, 75 µM Phos-Tag™ (NARD Institute LTD) and 150 µM $Zn(NO_3)_2$] (Boulanger-Castaing, A., and Hinton, D. M., personal communication). Purified protein was detected by staining the gel with SimplyBlue™ (Invitrogen), whereas protein from cell lysate was visualised by Western immunoblot with anti-His_6 antibody (CellSignaling). Cell lysis for Phos-Tag™ analysis was executed at 4°C with 2X-SDS loading buffer. Prior to transfer of the protein onto the membrane, the gel was incubated at room temperature with gentle shaking for 15 minutes in Towbin buffer (50 mM tris and 40 mM glycine) containing 1 mM EDTA to chelate the zinc and then for another 15 minutes in only Towbin buffer.

IN VITRO ACETYLATION

In vitro acetylation of RNA polymerase was conducted with two distinct acetyl donors: acetyl-CoA (AcCoA) and acetylphosphate (AcP).

AcCoA - *In vitro* acetylation of purified RNA polymerase with AcCoA and purified His6-YfiQ was carried out by mixing 1.5 μM YfiQ, 3.5 μM RNAP and 0.05 mM [^{14}C]-AcCoA in an *in vitro* acetylation buffer (50 mM Tris-HCl pH 8.0, 10% glycerol, 10 mM sodium butyrate, 1 mM DTT) to a final volume of 20 μL. The reaction was incubated for 1 hour at 37°C and stopped by the addition of NuPAGE 4X SDS loading buffer.

For visualization, the samples were heated at 95°C for 2 minutes, and resolved using a 12% acrylamide SDS-PAGE gel. After sample separation, the gel was dried for 2 hours using a gel dryer. Images were obtained by exposing a storage phospho screen to the dry gel overnight. The screen was read using a Typhon phosphoimager.

AcP - *In vitro* acetylation of purified RNA polymerase with AcP was carried out by mixing 3.5 μM RNAP with AcP at concentrations ranging from 0-40 mM in *in vitro* transcription buffer (40 mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 50 mM KCl, and 1mM DTT) and incubated for 1 hour at 30°C. After 1 hour, the reaction was stopped by the addition of standard 2X SDS loading buffer. Protein acetylation by AcP was detected by Western immunoblot analysis using an antibody against acetylated lysine (CellSignaling) at a ratio of 1:500.

IN VITRO TRANSCRIPTION

In vitro transcription of *cpxP* and all other promoters described in this dissertation was executed by multiple round transcriptions. The final volume of the *in vitro* transcription reaction was 25 µL and contained 1x *in vitro* transcription buffer (40 mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 50 mM KCl, 1mM DTT and 25 ng BSA), 1X NTP mixture (200 µM ATP, CTP and GTP, and 10 µM UTP), 2 µCi alpha [^{32}P]UTP, 50 ng *p770-cpxP* plasmid, purified His6-CpxR at concentrations ranging from 0.0-0.8 µM, AcP at concentrations ranging from 0 – 40 mM, and 10 nM of purified RNA polymerase-containing sigma 70 ($E\sigma^{70}$). When multiple reactions were performed per experiment, they were started at 30 second intervals by the addition of $E\sigma^{70}$ and incubated at 30°C for 15 minutes. After incubation, the reactions were stopped (also at 30-second intervals) by the addition of 25 µl of 2x stop solution (7 M Urea, 10 mM EDTA, 1% SDS, 2X TBE and 0.05% bromophenol blue). Immediately following the addition of the stop solution, the reactions were transferred onto ice.

The transcript fragments were separated using a denaturing acrylamide gel containing (6% acrylamide [19:1 acryl.:bis.], 7 M urea and 1x TBE). Polymerization was initiated by the addition of 0.01% of freshly made 10% APS solution and 0.001% TEMED and allowed to polymerize for 1 hour and 30 minutes undisturbed at room temperature.

23 µL of the *in vitro* transcription/stop solution reaction was loaded into the gel wells. The samples were allowed to run at approximately 200V for approximate-

ly 1.5-2 hours until the dye reached a position approximately 3 inches from the bottom of the gel, which keeps the unincorporated radioactivity on the gel and makes cleaning up the radioactive waste much easier. 1x TBE was used as the running buffer. Prior to phosphor imaging, the gels were dried using a gel drier for 2 hours. Images were obtained by exposing a storage phosphor screen to the dry gel overnight. The screen was read using a Typhon phosphoimager. The unused *in vitro* transcription reaction was stored at -20°C.

RNA POLYMERASE EXPRESSION AND PURIFICATION

RNAP expression - A single colony of BL21 (DE3) cells carrying plasmid *pIA900* was inoculated into 50 mL of LB containing 100 µg/mL ampicillin and incubated overnight at 37°C with aeration, rotating at 250 RPM. This overnight culture was used the following day to inoculate 1 L of LB containing 100 µg/mL ampicillin in a 2 L baffled flask, bringing the OD600 to 0.05. The culture was incubated at 37°C with aeration, rotating at 250 RPM until the culture OD_{600} reached 0.6, at which point 1 mM IPTG was added to induce RNA polymerase expression. RNAP expression was allowed to continue for approximately 6 hours under the same conditions before the culture was split into two 500 mL aliquots and the cells were pelleted by centrifugation at 10000 RCF for 10 minutes at 4°C. After centrifugation the supernatant was decanted and the cell pellets were placed at -20°C overnight or until cell lysis.

Protein extraction - For protein extraction, the cell pellets were allowed to thaw on ice. After thawing, 5 mL BugBuster (Novagen®) and 5 µL lysonase™ (Novagen®) was used to resuspend the cell pellets. After resuspension, the mixture were transferred to OakRidge tubes and incubated at room temperature for 30 minutes with gentle shaking. After incubation, 15 ml of RNAP resuspension buffer pH 8.0 was added (50 mM Na_2HPO_4, 1.4 M NaCl, 20 mM imidazole, 0.1% Tween 20, 5% ethanol and 15 mM β-mercaptoethanol). Cell debris was pelleted at 16000 RCF for 30 min at 4°C, after which the supernatant was collected for protein purification.

RNA Polymerase Purification – Purification of RNAP was conducted by passing the supernatant through a 1 mL cobalt resin bed that had been previously washed twice with 10 mL of water and equilibrated with 10 mL resuspension buffer. After the cell lysate was passed through the column, the column was washed with 10 mL wash buffer at pH 8.0 (50 mM Na_2HPO_4, 0.3 M NaCl, 30 mM imidazole, 0.1% Tween 20 and 5% ethanol). Protein elution was achieved by passing 3 mL of wash buffer containing 300 mM imidazole through the column. The eluted solution was collected and diluted into 3 mL TGED (0.01 M Tris-HCl pH 8.0, 5% glycerol, 0.1 mM EDTA, 0.1 M NaCl and 2 mM DTT). The diluted eluate was loaded onto 400 µL of heparin resin previously washed twice with 10 mL of water and equilibrated with TGED. The flow-through was collected and passed over the column a second time, after which the column was washed with 6 ml TGED containing 0.2 M NaCl, and RNAP was eluted from the column with 3 mL of TGED containing 0.6 M NaCl. The flow-through was transferred into a dialysis cassette and placed into 1 L dialy-

sis/storage buffer (10 mM Tris-HCl pH 8.0, 100 mM KCl, 50% glycerol, 10 mM $MgCl_2$, 0.1 mM EDTA and 1 mM DTT) overnight at 4°C with gentle stirring. After dialysis the samples were divided ino aliquots and stored at -80°C for future use.

CHAPTER THREE

RESULTS

ACETYLPHOSPHATE AS A PHOSPHORYL DONOR

In this chapter, I will address the hypothesis that AcP can function as a phosphoryl donor to RRs *in vivo*. Using a combination of genetic and biochemical approaches, I will specifically test the hypothesis that the RR CpxR can be phosphorylated by AcP *in vivo*, and that this phosphorylation event affects transcriptional regulation of the *E. coli* stress-responsive gene *cpxP*.

DOES *cpxP* TRANSCRIPTION RESPOND TO GENETIC MANIPULATIONS PREDICTED TO AFFECT INTRACELLULAR LEVELS OF AcP?

Linkage between AcP and several 2CST pathways has been proposed previously [reviewed in (144, 145)]. Only a few studies, however, have provided strong genetic evidence for this linkage *in vivo* (38, 119). As part of my efforts to map the impact of AcP on transcriptional regulation of *E. coli* promoters, I re-explored the relationship between AcP and the transcriptional regulation of *cpxP*, via genetic manipulation of the Pta-AckA pathway and the 2CST pathway CpxAR.

cpxP is the most sensitive of the more than 100 *E. coli* genes predicted to be regulated by phosphorylation of CpxR (29, 30). *cpxP* transcription requires the RR

CpxR and it is thought to be almost exclusively dependent on it (23), making *cpxP* transcription a good readout of CpxR phosphorylation.

Although CpxR is thought to be absolutely required for *cpxP* transcription, the cognate SK CpxA is not. *cpxP* transcription can still be induced in *cpxA* mutants when glucose is supplemented to rich medium (23), an observation that has contributed to the hypothesis that, in the absence of CpxA, CpxR could be phosphorylated by AcP (23).

Besides responding to the addition of glucose to rich medium, the CpxAR pathway has been shown to integrate and respond to multiple environmental cues, including entry into stationary phase, alkaline pH, attachment to hydrophobic surfaces, the presence of copper in the extracellular environment and the accumulation of pilus subunits in the periplasm [reviewed in (137)]. Therefore, before I could determine if AcP influenced *cpxP* transcription, I first characterized *cpxP* transcription both in the presence and absence of glucose and determined whether the previously reported response to glucose could be the summation of multiple signals or if it was due solely to the presence of AcP.

To assess *cpxP* transcription, I measured β-galactosidase activity from ϕ(*PcpxP-lacZ*) (23), which is carried by the hybrid bacteriophage λRS88 and present as a monolysogen in the λ*attB* site of the chromosome of WT cells (strain PAD282) and its isogenic mutants *ackA* (strain AJW2790), *ackA pta* (strain AJW2791), *cpxA* (strain PAD348), and *cpxR1* (strain PAD292) (30) (Table 1). The

cpxR1 allele has a polar effect on the downstream gene *cpxA*; thus, the resultant strain lacks both CpxR and CpxA (24).

I grew these strains in TB at 37°C, harvested cells at regular intervals, and compared their growth characteristics and β-galactosidase activities. Mutants lacking CpxA or CpxR exhibited growth characteristics indistinguishable from those of their WT parent. In contrast, mutants lacking AckA or both AckA and Pta, as reported previously (148), grew more slowly but to a similar density (**Fig. 6A**). Because of the difference in the growth rate, all data obtained subsequently were plotted versus OD600, although for some experiments, only the last time point is shown.

WT cells exhibited two peaks of *cpxP* activity; the first and smaller peak occurred in mid-exponential phase (OD600, 0.5) and reached about 13,000 Miller units, while the second and larger peak corresponded with entry into stationary phase (OD600, 1.0) and reached about 46,000 Miller units (**Fig. 6B**). Both behaviors depended upon CpxA and CpxR, as both *cpxA* and *cpxR* mutants exhibited low activity throughout growth. Intriguingly, *ackA* and *ackA pta* mutants had a hybrid profile; they exhibited little activity during exponential growth but displayed substantial activity during entry into stationary phase. The existence of two activity peaks led me to speculate whether there were two distinct stimuli, one associated with exponential growth and one associated with entry into stationary phase. To be consistent with previously published data, I focused on the increase in *cpxP* transcription as the cultures entered stationary phase.

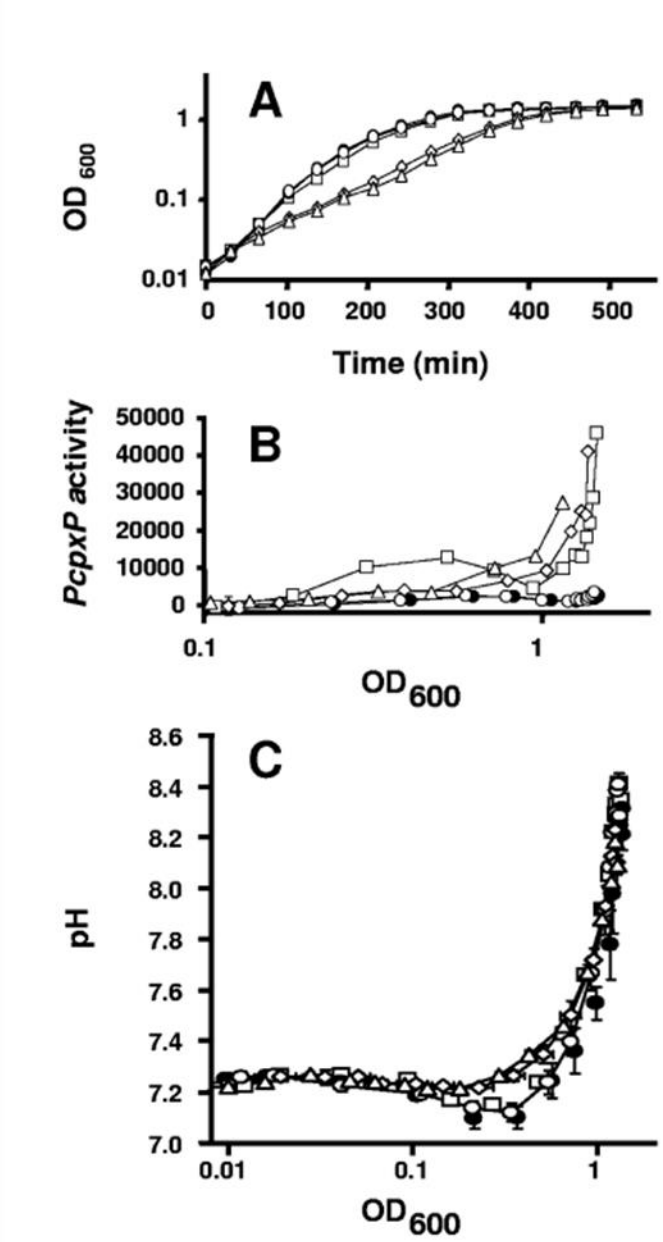


Fig. 6. FIG. 2. Disruption of the Pta-AckA pathway diminishes Cpx signaling. WT cells (strain PAD348) (open squares) and the isogenic *cpxA* (strain PAD348) (open circles), *cpxR* (strain PAD292) (closed circle), *ackA pta* (strain AJW2791) (open triangle), and *ackA* (strain AJW2790) (open diamond) mutants were lysogenized with a RS88 derivative that carried the (*PcpxP-lacZ*) transcriptional fusion. The resultant lysogens were grown with aeration in TBK at 37°C and harvested at regular intervals. The growth was monitored (A), the -galactosidase activity expressed in Miller units was plotted against OD600 to standardize for growth rate differences (B), and the culture pH was plotted against the OD600 (C). The values are the means standard deviations of triplicate independent cultures.

STATIONARY-PHASE BEHAVIOR IS A CpxA-DEPENDENT RESPONSE TO ALKALINE pH.

cpxP transcription has been reported to become activated in response to both elevated pH (23, 95) and entry into stationary phase (28, 30). Because TB is composed primarily of amino acids and because consumption of amino acids leads to the production of ammonia (104, 148), I expected that the culture pH would rise as the cells consumed amino acids and entered stationary phase and that the increased pH could lead to increased *cpxP* transcription. To test this hypothesis, I monitored the pH of the culture medium throughout growth and found that all five strains (WT, *cpxA, cpxR, ackA* and *ackA pta*) behaved almost identically (**Fig. 6C**). Each strain dramatically alkalinized its environment during the transition from exponential to stationary phase. In each case, the maximum pH reached by the culture was about 8.4. During mid-exponential growth, the *cpxA* and *cpxR* mutants and their WT parent slightly acidified their environments before alkalinizing them. The *ackA* and *ackA pta* mutants, in contrast, did not do this. This behavior can be attributed to the substantially reduced ability of mutants that lack a functional Pta-AckA pathway to generate and excrete acetate (104, 148).

Since the increase in pH was concomitant with entry into stationary phase, I performed two different experiments to distinguish between these two potentially distinct stimuli. First, I grew cells at 37°C in TB buffered at pH7 or TB buffered at pH8, harvested cells at regular intervals, and monitored their growth and *cpxP* activity. When grown at pH 8.0, WT cells exhibited about five times more promoter activ-

ity than they exhibited when they were grown at pH 7.0. In contrast, *cpxA* mutant exhibited weak promoter activity regardless of the pH (**Fig. 7B**). Since both types of cells grew more than twice as slowly at pH 8.0 as they did at pH 7.0 (**Fig. 7A**), it is possible that the higher activity at pH 8.0 could have been due to the lower growth rate instead of the higher pH. To differentiate between these two possibilities, I grew WT cells and *cpxA* mutants at 37°C in either unbuffered TB or TB buffered at pH7 (TB7). I harvested cells at regular intervals, and monitored their growth and *cpxP* activity (**Fig. 8**). WT cells exhibited high levels of *cpxP* activity in TB but not in TB7. In contrast, buffering had no significant effect on the *cpxP* activity exhibited by *cpxA* mutants. These results support the conclusion that pH is the primary stimulus behind the *cpxP* activity exhibited by WT cells as they enter stationary phase and, by extension, the isogenic *ackA* and *ackA pta* mutants. Furthermore, these data lend credence to the reports that the pH response requires CpxA (23, 95).

cpxP RESPONSE TO EXCESS CARBON REQUIRES THE Pta-AckA PATHWAY

It has been reported that exposure to exogenous glucose induces a CpxR-dependent *cpxP* transcription that depends on AcP (23, 24). However, the medium used to derive this conclusion was not buffered. Since I found that pH has a significant effect on *cpxP* transcription, I revisited this conclusion.

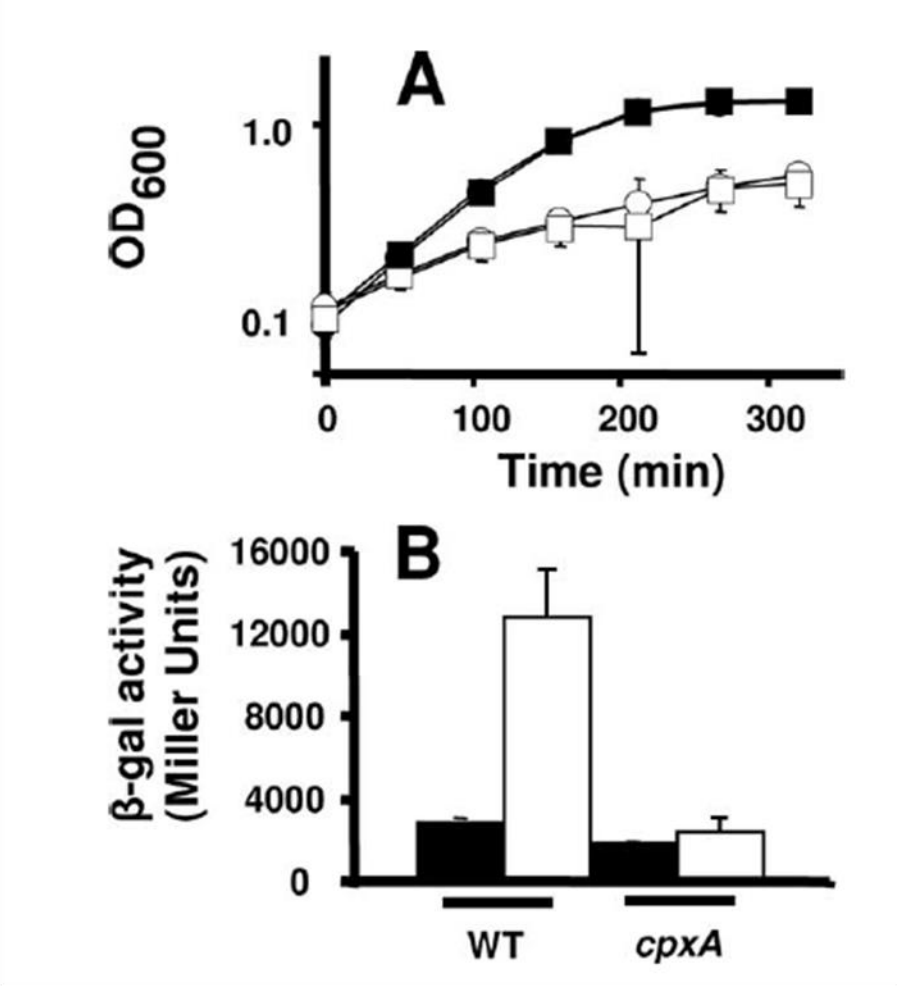


Fig. 7. Cpx response to alkaline pH requires CpxA. *cpxP* lysogens of WT cells (strain PAD282) (squares) or the isogenic *cpxA* mutant (strain PAD348) (circles) were grown with aeration at 37°C in TB& (filled symbols and filled bars) or TB8 (open symbols and open bars) and harvested at regular intervals. Growth was monitored (OD600) (A), and the -galactosidase (-gal) activity was plotted against OD600 (B). Only the final -galactosidase values are shown. The values are the means and standard deviations of triplicate independent cultures.

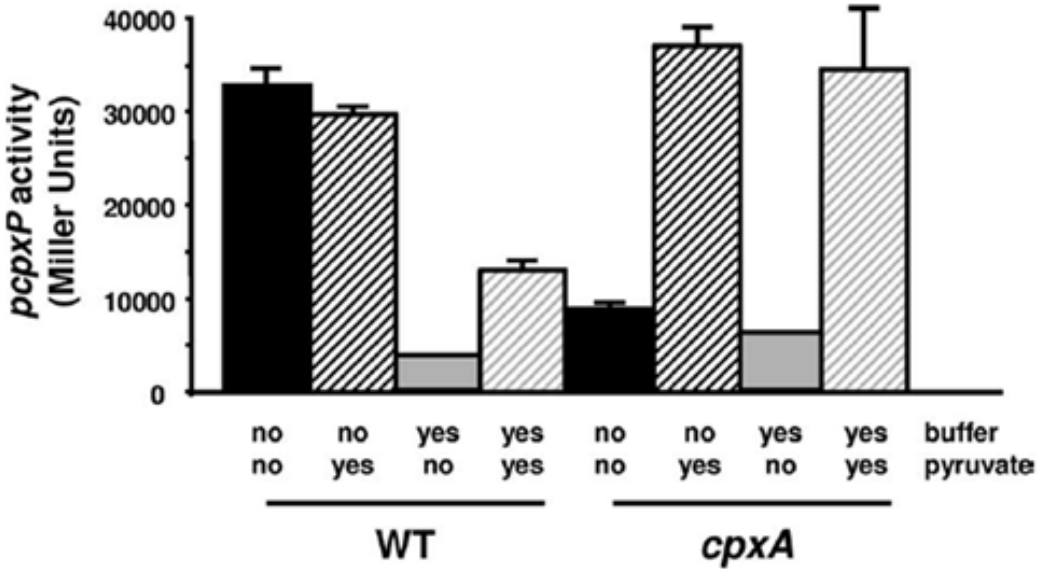


Fig. 8 Cpx response to pyruvate does not require CpxA. *pcpxP* lysogens of WT cells (strain PAD282) or the isogenic *cpxA* mutant (strain PAD348) were grown with aeration at 37°C in TB (black bars) or in TB7 (gray bars) either in the absence (filled bars) or in the presence (striped bars) of 0.8% sodium pyruvate. Cells were harvested at regular intervals, and the β-galactosidase activities were plotted against the OD600; only the final values are shown. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.

Exposure to pyruvate (a three-carbon glycolytic intermediate) results in greater accumulation of intracellular AcP than exposure to glucose (a six-carbon compound) (64, 72). Thus, to investigate the effect of AcP on *cpxP* transcription, I began by exposing cells to pyruvate. To be able to compare the *cpxP* responses to the two compounds, I standardized the concentration to the total number of carbons. Since 0.4% was the glucose concentration used previously (23, 24), I conducted my experiments with 0.8% sodium pyruvate. I grew WT cells and *cpxA* mutants at 37°C in pyruvate-supplemented TB7, harvested cells at regular intervals, and monitored their growth (data not shown) and *cpxP* activity (**Fig. 8**). Although WT cells responded to the presence of pyruvate, the total activity was less than one-half that of WT cells grown in unbuffered pyruvate-supplemented TB. In contrast, *cpxA* mutants responded strongly when they were exposed to pyruvate regardless of buffering. As reported previously for glucose (23), the *cpxA* mutant response to pyruvate was stronger than the response of its WT parent. Thus, in this context, CpxA inhibited the response to pyruvate, a behavior consistent with its reputed phospho-CpxR phosphatase activity (36, 111). Furthermore, my data corroborated previous observations that seem to support the proposition that CpxR can become activated in a CpxA-independent manner.

To determine if the CpxA-independent response to pyruvate requires the Pta-AckA pathway, I performed an epistasis analysis. I combined mutations in the Pta-AckA pathway with mutations in the CpxAR signaling pathway and compared the behavior of the cells in the presence and absence of pyruvate (**Fig. 9A**). WT cells

responded moderately (about threefold) to pyruvate and only upon entry into stationary phase. In contrast, the *cpxA* mutant cells responded early and dramatically; their *cpxP* activity increased about nine fold from exponential growth to entry into stationary phase. Thus, the inhibitory effect of CpxA appears to maintain low *cpxP* activity throughout exponential growth and to limit the response upon entry into stationary phase, at least in medium buffered at pH 7.0. Conversely, cells lacking CpxA and AckA or CpxA, AckA and Pta did not respond to pyruvate (**Fig. 9 B and C**). This supports the hypothesis that the response to pyruvate requires the Pta-AckA pathway.

CPXA-INDEPENDENT *cpxP* TRANSCRIPTION REQUIRES PTA-ACKA BUT DOES NOT CORRELATE WITH PREDICTED INTRACELLULAR ACP LEVELS

To activate *cpxP* activity in the absence of CpxA, CpxR must have an alternative source of phosphoryl groups. Since the pyruvate response requires the Pta-AckA pathway, a prime candidate is the pathway intermediate, AcP, which has been shown to act through at least one other 2CST RR (RcsB) to influence genes involved in flagellation and encapsulation (38). Furthermore, CpxR can use AcP as a phosphoryl donor *in vitro* (101, 111), and AcP has been proposed to be responsible for the CpxA-independent glucose response (6, 23, 24). To be consistent with previously published data, from this point on, I used glucose to induce *cpxP* transcription and to test the hypothesis that AcP is responsible for the CpxA-independent, glucose-induced *cpxP* transcription.

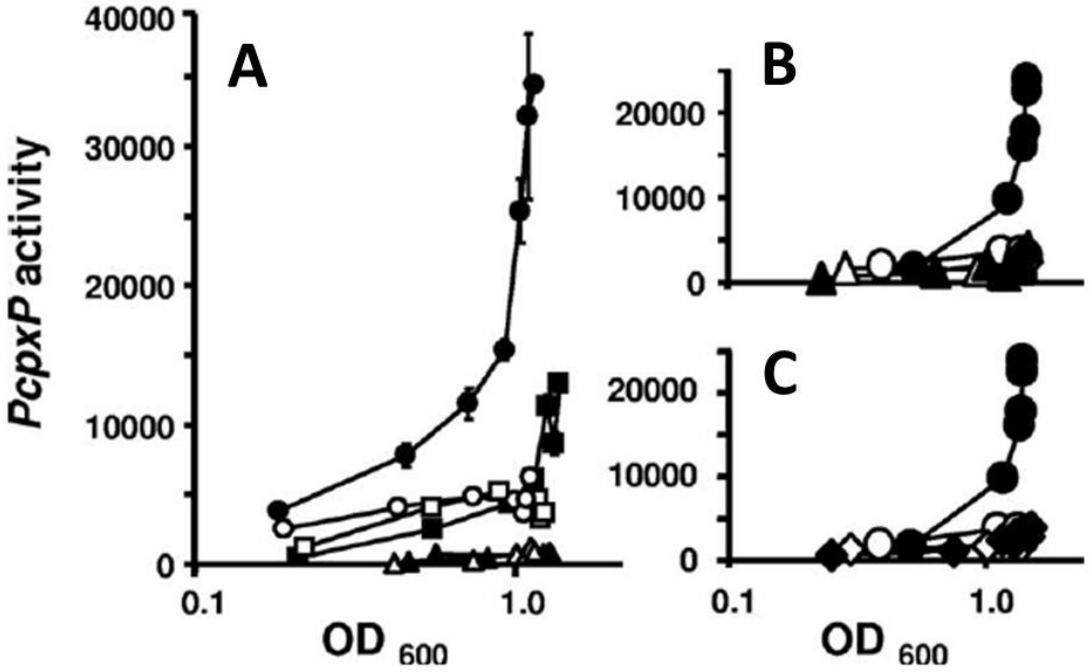


Fig. 9. (A) β-Galactosidase activities of *cpxP* lysogens of WT cells (squares) and mutants defective for either *cpxA* (circles) or *cpxR* (triangles) in the absence (open symbols) or presence (filled symbols) of 0.8% pyruvate plotted as a function of OD600. The values are the means standard deviations of triplicate independent cultures. (B) β-Galactosidase activities of *cpxP* lysogens of the *cpxA* mutant (circles) and the isogenic *cpxA pta ackA* mutant (triangles) in the absence (open symbols) or presence (filled symbols) of 0.8% pyruvate plotted against OD600. The values are the means of triplicate independent cultures. The standard deviations are smaller than the symbols. (C) β-Galactosidase activities of *PcpxP* lysogens of the *cpxA* mutant (circles) and the isogenic *cpxA ackA* mutant (diamonds) in the absence (open symbols) or presence (filled symbols) of 0.8% pyruvate plotted against OD600. The values are the means of triplicate independent cultures. The standard deviations are smaller than the symbols.

If AcP is the phosphoryl donor for CpxA-independent activity, then one would predict that the *cpxA ackA* double mutant, which accumulates AcP, would exhibit more activity than the *cpxA* mutant, while the *cpxA ackA pta* triple mutant, which cannot synthesize AcP, would exhibit less. While the latter is true, the former is not (**Fig. 10A**). As previously reported, and consistent with the role of CpxA as a phosphatase, *cpxA* mutants responded to glucose more strongly than its WT parent. Although the CpxA-independent response depended on the presence of the Pta-AckA pathway, cells that lacked CpxA and AckA exhibited a response that was weaker than that seen by the *cpxA* mutant and not the predicted stronger response. Therefore, I concluded that the response to glucose requires an intact Pta-AckA pathway. The lack of response by the *pta ackA* mutant argues that the Pta-AckA pathway is required and the response by the *ackA* mutant suggests that the response involves AcP, but the weakness of the response suggests that something else is involved.

To test the hypothesis that AcP does activate *cpxP* transcription, I took advantage of the fact *pta* mutants retain a functional AckA and that the Pta-AckA pathway is reversible. Thus, since AckA converts acetate to AcP, I could set AcP levels by controlling the amount of acetate added to the medium (64), and create a condition in which we could have AcP at a concentration that is predicted to be higher than WT, and yet, maintain a functional AckA enzyme.

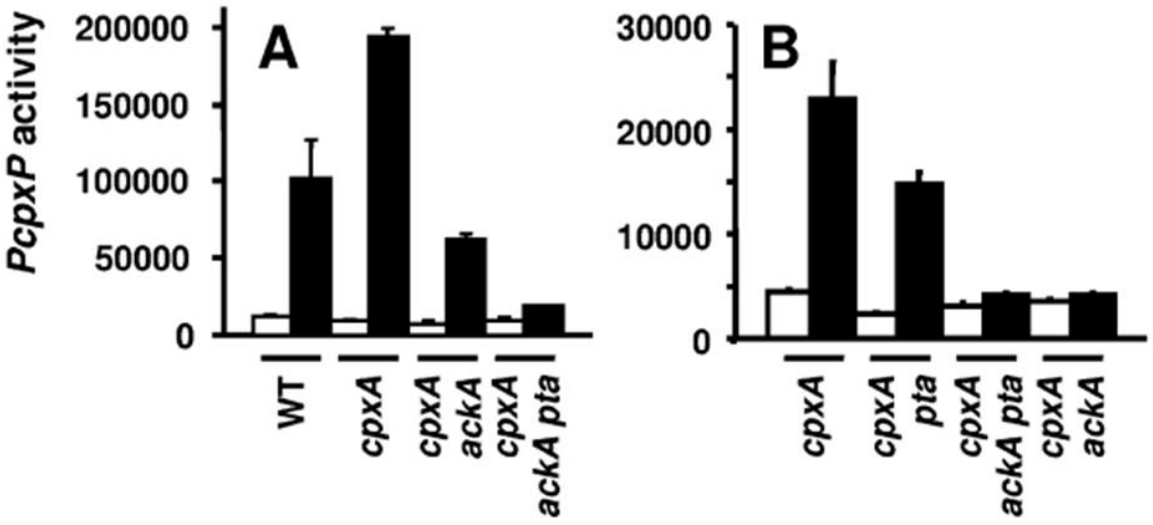


Fig. 10. Cpx response to glucose depends on the Pta-AckA pathway. (A) *cpxP* lysogens of WT cells (strain PAD282) and the isogenic *cpxA* (strain PAD348), *cpxA ackA* (strain AJW2794), *cpxA pta ackA* (strain AJW2970), and *cpxR* (PAD292) mutants were grown with aeration at 37°C in TB7 in the absence (open bars) or presence (filled bars) of 0.4% glucose and harvested at regular intervals, and the growth and β-galactosidase activity were monitored. Only the peak values are shown. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations. (B) *PcpxP* lysogens of the isogenic *cpxA* (strain PAD348), *cpxA pta* (strain AJW2964), *cpxA pta ackA* (strain AJW2970), and *cpxA ackA* (strain AJW2794) mutants were grown with aeration at 37°C in TB7 in the absence (open bars) or presence (filled bars) of 15 mM potassium acetate and harvested at regular intervals, and the growth and β-galactosidase activity were monitored. Only the peak values are shown. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.

To obtain that condition, I grew strains lacking CpxA and either AckA or Pta or both in TB7 or TB7 supplemented with 15 mM potassium acetate. Both the *cpxA* and *cpxA pta* mutants responded by exhibiting statistically significant *cpxP* activity when compare to cells grown in the absence of acetate, while the *cpxA ackA* and *cpxA ackA pta* mutants did not (**Fig. 10B**). Thus, I concluded that AcP can activate *cpxP* transcription. If so, then why is the response weaker when compared to cells that possess an intact Pta-AckA pathway? One possibility is that an inhibitor exists under conditions that presumably induce very high AcP concentration. To test this idea, I exposed *cpxA* mutants (which possess intact Pta-AckA pathway) to increasing concentrations of glucose. Consistent with the idea that an inhibitor of *cpxP* transcription accumulates under conditions that are expected to produce large AcP concentrations, I detected an increase in *cpxP* when cells were exposed to glucose concentrations up to 0.4%, but a progressive decrease in the presence of larger amounts, e.g. 4.0% (**Fig. 11**).

On the basis of these results, I conclude that AcP contributes to *cpxP* transcription, perhaps via the transfer of a phosphoryl group from AcP to CpxR, as such a transfer has been shown to occur *in vitro* (85, 101). Since *cpxP* transcription fails to strictly correlate with expected intracellular levels of AcP, it is possible that AcP is not the sole contributor to CpxA-independent, glucose-induced *cpxP* transcription. In this dissertation, I will first ask if a transfer of phosphoryl groups occurs between AcP and CpxR and later ask whether factors other than CpxR phosphorylation contribute to the CpxA-independent, glucose-induced transcription of *cpxP.*

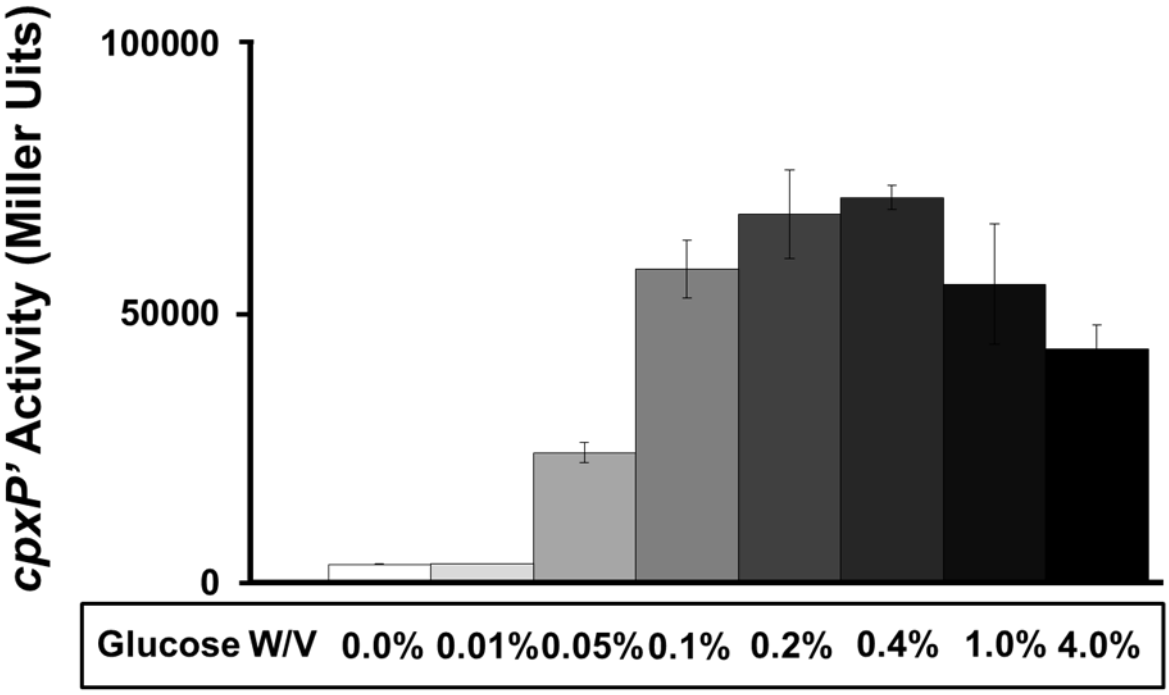


Fig. 11. Excessive glucose inhibits cpxP transcription.
λ*cpxP* lysogens of the *cpxA* mutant (strain PAD348) were grown for 7.5 hours at 37°C with shaking in TB7 supplemented with increasing concentrations of glucose. Cells were harvested at regular intervals and OD600 and β-galactosidase activity were measured. Only the values at the last time point are shown. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.

GLUCOSE-INDUCED *cpxP* TRANSCRIPTION REQUIRES ASP-51, THE CONSERVED PHOSPHORYL ACCEPTOR RESIDUE OF CPXR

The knowledge that glucose-induced *cpxP* transcription is affected by AcP, that it does not require the sensor kinase CpxA, but that it requires the cognate response regulator CpxR (149), led me to re-investigate the role of CpxR phosphorylation. To address this question, I attempted to complement the *cpxR1* mutant allele in a strain that carries the transcriptional fusion Φ(*PcpxP'-lacZ*) (strain PAD 292; **Table 1**). Complementation was attempted by transformation with either plasmid-borne WT *cpxR* (pCA24n-*cpxR*; **Table 1**) or plasmid-borne *cpxRD51A* (pCA24n-*cpxRD51A*; **Table 1**). The latter encodes a mutant protein that lacks the conserved aspartyl residue that serves as the phospho-acceptor site. Both alleles were expressed from an IPTG-inducible promoter. I grew the resultant transformants in TB with or without the presence of 0.4% glucose and measured β-galactosidase activity as a reporter of *cpxP* promoter function. The transformants that expressed WT CpxR responded to glucose. In contrast, the transformants that expressed CpxRD51A did not (**Fig. 12, left**). Since Western immunoblot analysis indicated that the steady state levels of the WT and mutant forms of CpxR proteins were similar (**Fig. 12, inset**), the lack of *cpxP* transcription by cells that express the mutant protein cannot be explained by a difference in protein expression levels. Instead, this lack of response likely resulted from the mutant protein's inability to become phosphorylated (**Fig. 13B**). I therefore conclude that the response to glucose by *cpxP* requires AsP-51 and propose that this response requires phosphorylation of CpxR.

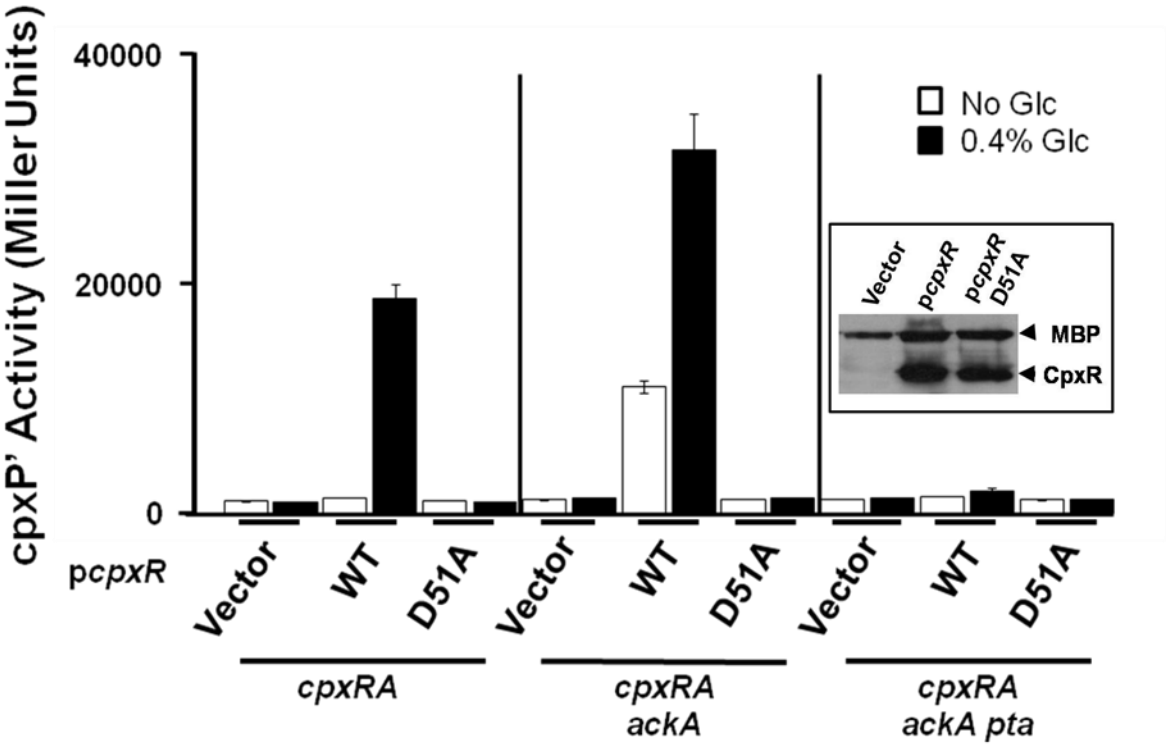


Fig. 12. The glucose response requires D51 and AcP.
A) λ*cpxP* lysogens of the *cpxR1* mutant (strain PAD292), the *cpxR1 ackA* mutant (strain AJW3827), and the *cpxR1 ackA pta* mutant (strain AJW3875) were transformed with the plasmids pCA24n (vector), pCA24n-*cpxR* (WT), or pCA24n-*cpxR-D51A* (D51A). Transformants were grown at 37°C with shaking in TB7 (open bars), or the same medium supplemented with 0.4% glucose (closed bars). Cells were harvested at regular intervals and OD_{600} and β-galactosidase activity were measured. Only the values at the last time point are shown. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.
(Inset) Western immunoblot analysis of steady state levels of plasmid-expressed CpxR and CpxR-D51A from whole-cell lysates of PAD292 using polyclonal antibody generated against MBP-CpxR fusion protein (gift from Thomas Silhavy). Endogenous MBP serves as a loading control.

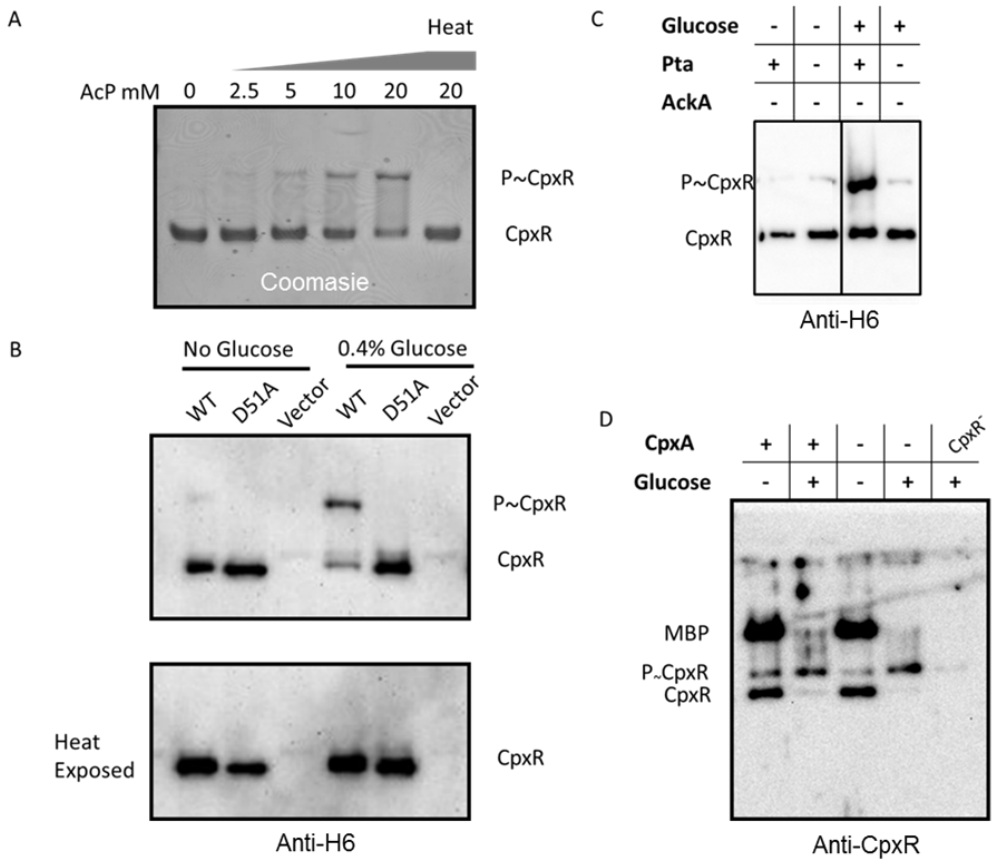


Fig. 13. Detection of phosphorylated CpxR.

A) Purified His_6-CpxR was incubated with increasing concentration of AcP at 30°C for 15 minutes and then resolved on a Phos-Tag™ SDS-PAGE. A fraction of the *in vitro* phosphorylation reaction carried out with 20 mM AcP was incubated at 95°C for 15 minutes to promote hydrolysis of the phosphoryl group. The protein was detected by coomassie brilliant blue.

B) λ*cpxP* lysogens of the *cpxR1* mutant (strain PAD292) transformed with plasmids pCA24n (vector), pCA24n-*cpxR* (WT), or pCA24n-*cpxR-D51A* (D51A) were grown in TB7 in the absence or presence of 0.4% glucose at 37°C with shaking. 50 mM IPTG was added to the cultures to induce CpxR expression after cultures reached an OD_{600} of 0.6. After 3 hours of induction, cell lysates were collected and resolved by Phos-Tag™ SDS-PAGE before and after incubation at 95°C for 15 minutes. CpxR was visualized by Western immunoblot with anti-His_6 antibody.

C) λ*cpxP* lysogens of the *cpxR1 ackA* mutant (strain AJW3827), and the *cpxR1 ackA pta* mutant (strain AJW3875) transformed with the plasmid pCA24n-*cpxR* were grown in TB7 in the absence or presence of 0.4% glucose at 37°C with shaking. After cultures reached an OD_{600} of 0.6, 50 mM IPTG was added to the cultures to induce CpxR expression. After 3 hours of induction, cell lysates were collected and resolved by Phos-Tag™ SDS-PAGE. CpxR was visualized by Western immunoblot with anti-His_6 antibody.

D) λ*cpxP* lysogens of the WT strain (strain PAD282), the *cpxA* mutant (strain PAD348), and the *cpxR1* mutant (strain PAD292) were grown in TB7 in the absence and presence of 0.4% glucose at 37°C with shaking for 7.5 h. Cell lysate was collected and resolved by Phos-Tag™ SDS-PAGE. Endogenous WT CpxR was detected using polyclonal antibody generated against the MBP-CpxR fusion protein. Note that MBP is catabolite-repressed. Therefore, its expression is inhibited in the presence of glucose.

THE RESPONSE TO GLUCOSE REQUIRES ACETYL PHOSPHATE

The knowledge that CpxA-independent, glucose-induced *cpxP* transcription seems to be, at least in part, dependent on AcP and seems to require phospho-CpxR led me to investigate if the weak response to glucose by the *ackA* mutant required CpxR phosphorylation, perhaps an indicator that AcP could indeed function as a donor of phosphoryl groups to CpxR.

If AcP can function as a phosphoryl donor to CpxR, then the *cpxR1 ackA* double mutant (strain AJW3827; **Table 1**), which accumulates AcP (72, 146), should respond to the addition of glucose in a Asp-51-dependent manner. In contrast, I predicted that the *cpxR1 pta ackA* triple mutant (strain AJW3875; **Table 1**), which cannot synthesize AcP (72, 146), would not respond to exogenous glucose regardless of the status of Asp-51. Indeed, the *cpxR1 ackA* double mutant responded to glucose in an Asp-51-dependent manner, while the *cpxR1 pta ackA* triple mutant did not respond to glucose (**Fig. 12A, middle and right respectively**). Thus, in the absence of CpxA and in the presence of glucose, *cpxP* transcription requires both Asp-51 and AcP.

IN VIVO PHOSPHO-CpxR LEVELS CORRELATE WITH AcP BUT NOT WITH *cpxP* TRANSCRIPTION

The simplest explanation for the requirement of both AcP and Asp-51 is that the CpxA-independent, glucose-induced *cpxP* transcription requires an AcP-dependent phosphorylation of CpxR. Despite this requirement for AcP, the AcP-

accumulating *ackA* mutant fails to promote the predicted proportional increase in *cpxP* transcription (**See Figures 10A, B and 12**). To understand this paradoxical behavior, we assessed whether *in vivo* levels of phospho-CpxR correlate with those of AcP in strains WT or mutant for the Pta-AckA pathway.

To monitor phospho-CpxR, I took advantage of a previously reported method used for the detection of phosphorylated proteins (69, 70), including bacterial response regulators (5, 85). This method uses a dinuclear metal complex (*i.e.* 1,3-bis [bis (pyridin-2-ylmethyl) amino] propan-2-olato dizinc [II]) that has affinity for phosphomonoester dianions, such as the aspartyl phosphate of response regulators. When included in an SDS-PAGE gel, this phosphate-binding tag (Phos-Tag™) slows the migration of the phosphorylated protein, allowing it to be distinguished from the non-phosphorylated protein by mobility shift.

To verify the report that AcP can function *in vitro* as a phosphoryl donor to CpxR (101) and to optimize the assay, I incubated purified His_6-CpxR with AcP, resolved the proteins by Phos-Tag™ SDS-PAGE and stained the gel with SimplyBlue™ (Invitrogen). I detected an AcP-dependent shift in CpxR migration that disappeared when the sample was exposed to 95°C for 15 minutes, a condition that induces hydrolysis of phosphorylated aspartyl residues (**Fig. 13A**).

To test the hypothesis that glucose induces *cpxP* transcription by increasing phospho-CpxR concentration, I assessed the *cpxR1* mutant (strain PAD292) transformed with plasmids that expressed either His_6-CpxR or His_6-CpxR D51A. I grew the transformants in the absence or presence of 0.4% glucose, harvested cells as the

cultures entered stationary phase, separated the cell lysates by Phos-Tag™ SDS-PAGE and performed a Western immunoblot analysis with anti-His_6 antibody. Consistent with the idea that glucose-induced *cpxP* transcription requires phospho-CpxR, I detected a glucose- and Asp-51-dependent shift in CpxR that was sensitive to heat (**Fig. 13B**).

To test the role of AcP, I performed a similar experiment on *cpxR1 ackA* (strain AJW3827, **Table 1**) and *cpxR1 pta ackA* (strain AJW3875, **Table 1**) mutants transformed with the His_6-CpxR expression plasmid. Consistent with the hypothesis that AcP mediates CpxR phosphorylation, the shifted band was absent in the cells that cannot synthesize AcP (*cpxR1 pta ackA*) but present in the cells that accumulate AcP (*cpxR1 ackA*) (**Fig. 13C**).

To facilitate detection of shifted phospho-CpxR, the aforementioned Phos-Tag™ experiments were performed with cells that overexpressed His_6-CpxR. Because I was aware that overexpression of tagged proteins often causes artifacts, I performed a similar experiment with WT and *cpxA* mutant cells that expressed WT CpxR from the native locus. In response to glucose, the phosphorylated fraction of native CpxR increased substantially, regardless of the status of CpxA (**Fig. 13D**).

CONCLUSION

AcP has been proposed to play a role in transcription regulation by serving as a phosphoryl group donor to several RRs. In this chapter, I tested this hypothesis using the CpxAR system, which had been proposed previously to be influenced by an AcP-dependent phosphorylation of the RR CpxR.

My results demonstrate that the addition of glucose, pyruvate and acetate to TB7 can induce *cpxP* transcription even in the absence of the cognate sensory protein CpxA. Genetic manipulations of the Pta-AckA pathway and of the CpxAR 2CST demonstrated that glucose-induced (and to some degree pyruvate-induced) *cpxP* transcription requires the Pta-AckA pathway, CpxR and its phosphorylation site, Asp-51. The simplest explanation to these results is that, in the absence of CpxA and in the presence of glucose, and likely other carbon sources, AcP can function as the phosphoryl donor to CpxR, and that this phosphoryl transfer leads to transcription activation of *cpxP.* The conclusions derived from my genetic experiments were supported by biochemical data that demonstrate that *in vivo* phosphorylation of CpxR requires glucose, AcP and the phosphorylation site, Asp-51. This was the first time that biochemical evidence demonstrated that a RR is phosphorylated *in vivo* in an AcP-dependent manner and showed that the AcP-dependent phosphorylation site is the same as the one phosphorylated by the cognate sensor kinase-dependent mechanism. While these results make a straightforward case for the transfer of phosphoryl group from AcP to CpxR, the weak response to glucose by *ackA* mutants, de-

spite the presence of phosphorylated CpxR, suggests that regulation of *cpxP* transcription involves more than just CpxR phosphorylation.

In the following chapter, I will address the hypothesis that regulatory mechanisms, other than CpxR phosphorylation, are involved in the CpxA-independent, glucose-induced *cpxP* transcription.

CHAPTER FOUR

RESULTS

ACETYLATION AND TRANSCRIPTIONAL REGULATION

In the previous chapter, I provided evidence that supports the hypothesis that, upon the addition of 0.4% glucose to TB, AcP phosphorylates CpxR *in vivo* and that this phosphorylation event impacts *cpxP* transcription. However, *cpxP* transcription does not fully correlate with the predicted intracellular concentration of AcP (64). While I did find that *pta ackA* double mutants, which do not synthesize AcP, failed to induce *cpxP* transcription, *ackA* mutants (which are predicted to accumulate more AcP than WT) exhibited *cpxP* transcription below WT levels, even though CpxR was found to be phosphorylated under the same conditions. This somewhat paradoxical behavior led me to hypothesize that additional regulatory mechanism(s) might modulate the CpxA-independent, glucose-induced and AcP-dependent *cpxP* transcription.

Through a series of genetic and biochemical approaches I investigated whether, in addition to CpxR phosphorylation, other regulatory mechanism could regulate *cpxP* transcription. The data that I will present here implicate protein acetylation in a sophisticated regulatory mechanism that seems to modulate AcP-dependent *cpxP* transcription.

CATABOLITE REPRESSION

In *E. coli* and many other bacteria, glucose has a profound impact on central metabolism, affecting, among other things, the concentration of cyclic-AMP. Cyclic-AMP is an important transcriptional regulator that modulates the activity of CRP, the master regulator of *E. coli* central metabolism. By binding to CRP, cyclic-AMP induces a conformational change that allows CRP to interact with many promoter regions and to regulate their activity. Glucose, however, leads to a decrease in total cyclic-AMP concentration, preventing CRP from binding DNA [reviewed in (144)]. Although there is no evidence that this event (known as catabolite repression) could affect *cpxP* transcription via a direct interaction with the *cpxP* promoter region, there was no evidence to rule out an indirect effect.

To test whether catabolite repression could influence *cpxP* transcription, I exposed *cpxA* mutants (Strain PAD348 **Table 1**) to a non-catabolite-repressing carbon source (acetate) and to two catabolite-repressing carbon sources (glucose and pyruvate), measured β-galactosidase activity and compared the *cpxP* response to each of these carbon sources (**Fig. 14**). Since glucose, pyruvate and acetate induced similar responses, it is unlikely that catabolite repression plays a substantive role in regulating *cpxP* transcription. Therefore, I explored other effects that exposure to glucose might exert on *E. coli* physiology.

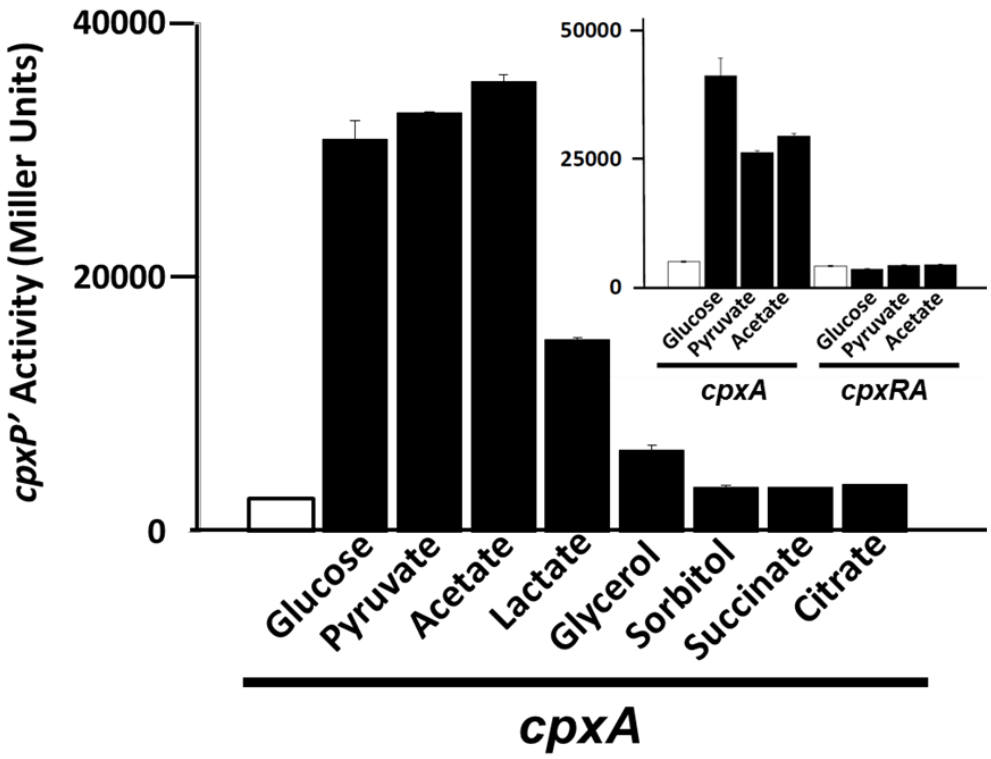


Fig. 14. Diverse carbon sources can induce CpxA-independent *cpxP* transcription.
A λ*cpxP* lysogen of the *cpxA* mutant (PAD348; Table 2) was grown at 37°C with shaking in buffered TB (open bars) or in the same medium supplemented with 0.4% glucose, 0.8% pyruvate, 15 mM acetate, 0.8% lactate, 0.8% glycerol, 0.4% sorbitol, 0.6% succinate or 0.4% citrate (closed bars). Cells were harvested after 7.5 hours incubation and β-galactosidase activity was measured. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations. **Inset:** β-galactosidase activity of *cpxA* (PAD348) and *cpxRA* (PAD292; Table 1) mutants grown under the conditions described above.

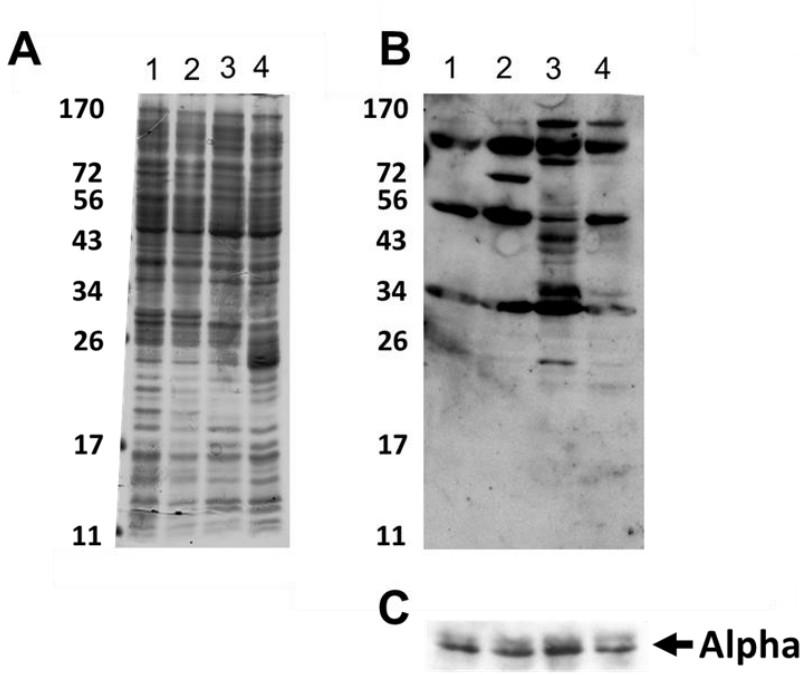


Fig. 15. The *E. coli* acetylome is sensitive to conditions that affect CpxA-independent *cpxP* transcription.

A λ*cpxP* lysogen of the wild-type strain (PAD 282) transformed with the vector pCA24n was grown at 37°C with shaking in buffered TB supplemented with 5 µM IPTG (column 1), the same medium supplemented with 50 mM nicotinamide (column 2), or 0.4% glucose (column 3). The same strain transformed with a pCA24n derivative carrying the *cobB* ORF was grown in buffered TB supplemented with 5 µM IPTG and 0.4% glucose (column 4). Cells were pelleted after 7.5 hours incubation and protein was harvested as described in the Materials and Methods. In each lane, 150 µg of protein was separated by SDS-PAGE. Panels show: A) Coomassie stain, B) Western immunoblot with cocktail of anti-acetyllysine polyclonal antibodies, and C) as a loading control, a Western immunoblot using anti-α monoclonal antibody.

GLUCOSE AND AcP AFFECT GLOBAL PROTEIN ACETYLATION

Besides lowering cyclic-AMP levels, glucose rapidly increases the AcCoA concentration in *E. coli* (21) and, recently, it has been shown to promote protein acetylation in *S. enterica* (139). *S. enterica,* however, is not the only bacterial species where protein acetylation occurs; global proteomic studies using mass spectrometry analysis have demonstrated that protein acetylation is common also in *E. coli* and *B. subtilis* (68). The studies in *E. coli* identified acetylated proteins that are part of many physiological processes. Of great interest to me was evidence that some of the RNA polymerase subunits (α, β and β') could be acetylated. Therefore, I considered the possibility that, in addition to protein phosphorylation, an acetylation event could influence *cpxP* transcription.

To test this hypothesis, I first asked whether, similarly to *S. enterica,* global protein acetylation in *E. coli* could be affected by the addition of glucose to the growth medium. To that end, I used a cocktail of two polyclonal anti-acetyllysine antibodies to perform Western immunoblot analysis on whole cell lysates from *cpxA* mutant cells grown either in TB7 supplemented with 0.4% glucose or not (**Fig 15**). As reported for *S. enterica* (139), the presence of glucose resulted in a general increase in the acetylome, as detected by the increase in number and/or intensity of acetylated bands. Subsequently, these results were verified and extended by Bozena Zemaitatis (a Research Specialist in our laboratory), who optimized the anti-acetyllysine Western immunoblot analysis, and quantified by our collaborators Birgit

Schilling and Bradford Gibson (Buck Institute for Aging, Novato, CA), who used a novel mass spectrometric approach.

To determine whether glucose enhanced the acetylome, Bozena grew MC4100 strains in TB7 and TB7 supplemented with 0.4% glucose, harvested cells at 5 time points over the course of two days (OD_{600} = 0.5 and 1.0 and 8, 24 and 32 hours), prepared cell lysates, and subjected them to anti-acetyllysine Western immunoblot analysis (**Fig. 16B**). While she observed more bands across the entire growth curve under both growth conditions, Bozena came to the same general conclusion: glucose enhances the WT acetylome. These results were verified and quantified by Drs. Schilling and Gibson, who applied mass spectrometry analysis to whole cell lysates from WT cells grown in TB7 or in TB7 supplemented with 0.4% glucose to OD_{600} = 1.0. In the absence of glucose, they detected 414 unique acetyllysines in 194 proteins in the WT parent. In presence of glucose, they detected 965 unique acetylation sites in 368 proteins. Thus, glucose enhances both the number of acetylations and the number of acetylated proteins.

To determine if the Pta-AckA pathway could affect protein acetylation, Bozena also performed anti-acetyllysine Western immunoblot analysis on cell lysates from *ackA* and *pta ackA* double mutants grown over two days in TB7 (without glucose supplementation). Relative to its WT parent, the *ackA* mutant was hyperacetylated, especially during exponential growth (**Fig. 16A**). In contrast, the acetylome of the *pta ackA* mutant more closely resembled that of its WT parent. These data suggested that the enhanced acetylation exhibited by the *ackA* mutant was due

to the accumulation of AcP, but it also could be a direct result of the enzyme Pta. To distinguish between these two hypotheses, Bozena tested a *pta* mutant (which retains AckA and thus the ability to accumulate AcP when exposed to exogenous acetate). When cells were grown in TB7 supplemented with 10 mM acetate, the acetylome was considerably more robust (**Fig. 16C**). Taken together, these data implicate AcP as a regulator of acetylation. Since growth in the presence of glucose not only increases the concentration of AcCoA, but also that of AcP (64), Bozena asked whether glucose-induced acetylation required AcP. Indeed, the increased acetylation exhibited by WT cells when grown in TB7 supplemented with glucose was completely absent in the *pta ackA* mutant (**Fig. 16B**). These results were verified by mass spectrometry. In the absence of glucose, our collaborators detected 414 acetylation sites in 194 proteins from WT lysates, 932 acetylation sites in 379 *ackA* proteins from *ackA* mutant lysates, but only 225 acetylation sites in 125 proteins from the *pta ackA* mutant. In the presence of glucose, mass spectrometry detected 965 acetylated sites in 368 proteins from the WT parent but 2150 unique acetylation sites in 664 proteins from whole cell lysates derived from the *ackA* mutant. They did not analyze the *pta ackA* mutant grown in the presence of glucose.

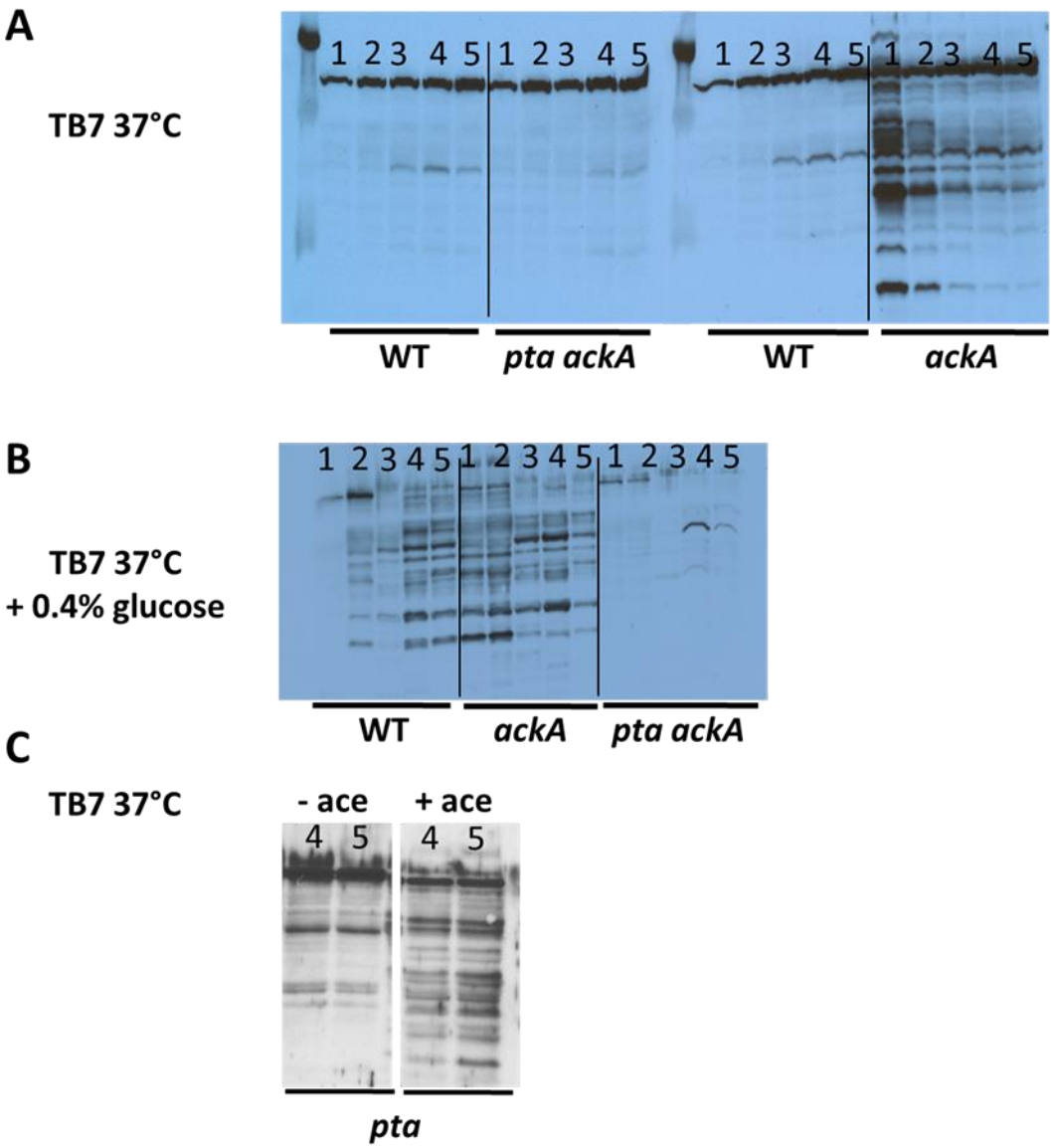
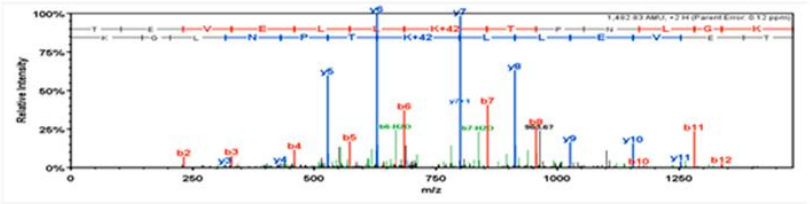


Fig. 16. AcP Contributes to Global Protein Acetylation.
WT, *ackA, pta ackA and pta* mutant cells were grown in TB7 (A) without glucose, (B) with 0.4% glucose, (C) or with or without 10 mM acetate. The cells were harvested at **1**- OD600 = 0.5, **2**- OD600 = 1.0, **3**- after 8hs of incubation, **4**- after 24hs of incubation and **5**- after 32 hs of incubation. The proteins were resolved using a SDS-PAGE gel, transferred to a membrane and proteins acetylation was detected using a anti-acetylated lysine antibody cocktail.

A

Peptide sequence	Peptide identification probability	SEQUEST XCorr score	SEQUEST ΔCn score	m/z precursor ion	Peptide neutral molecular mass	Charge	Mass deviation (ΔPPM)
M_1(+16)QGSVTEFLK$_{10}$(+42)PR(L)	95%	3.9936	0.6951	725.873	1449.7315	2	1.923
(R)LVDIEQVSSTHAK$_{25}$(+42) VTLEPLER(G)	95%	4.5772	0.7147	802.7703	2405.2891	3	0.119
(R)VQGK$_{95}$(+42)DEVILTLNK(S)	95%	3.4690	0.5055	749.9274	1497.8403	2	-0.154
(R)TEVELLK$_{291}$(+42)TPNLGK(K)	95%	3.5546	0.6862	742.4222	1482.898	2	0.115
(K)TPNLGK$_{297}$(+42)K(S)	95%	2.4231	0.4308	400.2372	798.4594	2	-0.863
(K)SLTEIK$_{304}$(+42)DVLASR(G)	95%	3.3245	0.5804	687.3862	1372.7563	2	1.056

B (R)TEVELLK$_{291}$(+42)TPNLGK(K)



C

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	102.1			84.0	T+42	1,483.8	742.4	1,466.8	1,465.8	13
2	231.1			213.1	E	1,382.8	691.9	1,365.8	1,364.8	12
3	330.2			312.2	V	1,253.7	627.4	1,236.7	1,235.7	11
4	459.2			441.2	E	1,154.7	577.8	1,137.7	1,136.7	10
5	572.3			554.3	L	1,025.6	513.3	1,008.6	1,007.6	9
6	685.4	343.2		667.4	L	912.6	456.8	895.5	894.5	8
7	855.5	428.2	838.5	837.5	K	799.5	400.2	782.4	781.5	7
8	956.5	478.8	939.5	938.5	T	629.4	315.2	612.3	611.4	6
9	1,053.6	527.3	1,036.6	1,035.6	P	528.3		511.3		5
10	1,167.6	584.3	1,150.6	1,149.6	N	431.3		414.2		4
11	1,280.7	640.9	1,263.7	1,262.7	L	317.2		300.2		3
12	1,337.7	669.4	1,320.7	1,319.7	G	204.1		187.1		2
13	1,483.8	742.4	1,466.8	1,465.8	K	147.1		130.1		1

Fig. 17. In the glucose-exposed *ackA* mutant, the α subunit of RNAP is acetylated on K291 and five other lysines.

Immunoprecipitated subunits of RNAP were separated *by SDS-PAGE. The α* bands were excised and tryptically digested as described previously (53). The resulting peptides were analyzed in a LTQ OrbitrapXL™ mass spectrometer as described in the Materials and Methods. The double- or triple-charged acetyllysine-modified peptides M1(+16)QGSVTEFLK10(+42)PR, LVDIEQVSSTHAK25(+42)VTLEPLER, VQGK95(+42)DEVILTLNK, TEVELLK291(+42)TPNLGK, TPNLGK297(+42)K, and SLTEIK304(+42)DVLASR were detected in the digested α sample as mass peaks of m/z=725.873, m/z=802.7703, m/z=749.9274, m/z=742.4222, m/z=400.2372 and m/z=687.3862, respectively. A) The Xcorr and ΔCn scores of the six acetylated peptides. B) The corresponding CID MS/MS spectrum for the K291-containing peptide TEVELLK291(+42)TPNLGK. C) Complete b and y fragment ion series for the K291-containing peptide TEVELLK291(+42)TPNLGK.

Among some of the proteins identified as differentially acetylated between WT and *ackA* mutants are all of the subunits of RNA polymerase. If RNA polymerase is indeed acetylated, one could imagine that its acetylation could affect transcription of many *E. coli* promoters, including *cpxP.* Therefore, I hypothesized that RNAP acetylation affects the CpxA-independent, AcP-dependent *cpxP* transcription.

To verify that RNAP subunits are indeed acetylated, I grew the *cpxA* mutant and *cpxA ackA* double mutant (strain AJW 2794, **Table 1**) in the presence or absence of glucose, purified RNAP subunits α, β and β' by immunoprecipitation, and sent the purified proteins to another collaborator (Dr. Haike Antelman) for mass spectrometry analysis.

From *ackA* cells grown in the presence of glucose, Dr. Antelmann and her group mapped 19 acetylation sites to β (**Table 2**), of which 12 were detected in at least two biological replicates. They mapped an additional 6 acetylation sites to β', of which 4 were detected in two biological replicates (**Table 2**). And finally, they mapped 7 acetylation sites to α, of which 4 were detected in two biological replicates (**Table 3, Fig. 17)**. None of the reproducibly acetylated α sites was acetylated when *ackA* cells were grown in the absence of glucose (**Tables 2 and 3)**. Dr. Antelmann's group also detected glucose-dependent acetylation on RNAP subunits purified from WT cells, but the acetylation sites differed from those detected in the *ackA* mutant (**Table 4**). I will first describe my efforts to determine if acetylation could be responsible for the weak response to glucose exhibited by *ackA* mutants. Later, I will describe my investigation into the role that RNAP acetylation plays in WT cells.

LYS-291 ON RNAP αCTD CONTRIBUTES TO TRANSCRIPTION INHIBITION

To test the hypothesis that the weak response to glucose by *ackA* mutants requires one of the acetylated lysines of RNAP, I used site-directed mutagenesis to convert the acetylated lysines to alanine; therefore, preventing their acetylation. Because the gene that encodes α (*rpoA*) is essential for bacterial survival, I used a previously reported partial-diploid system (39) to test the hypothesis that one of the acetylated lysines on α inhibits *cpxP* transcription. This system keeps the WT copy of *rpoA* at its native site in the chromosome, and introduces mutant *rpoA* alleles into the cell via a multi-copy plasmid allowing one to determine if any given residue on α is involved in a particular behavior.

Into an *ackA cpxA* Φ(*PcpxP'-lacZ*) lysogen that carries the WT *rpoA* gene in its native location on the chromosome (strain AJW2794), I introduced plasmids carrying either the WT *rpoA* allele or K-to-A point mutation derivative alleles (**Table 1**) with the goal of determining if any lysine functional group contributes to the weak response to glucose. I grew the resultant transformants in the absence or presence of 0.4% glucose and monitored β-galactosidase activity. *cpxA ackA* mutant cells that overexpressed the α K291A mutant responded robustly to the presence of glucose. In contrast, we observed little or no response from *cpxA ackA* mutants that overexpressed WT α, K-to-A mutations of the other 4 lysines of the αCTD (**Fig. 18A**) or K-to-A mutations of the 3 acetylated lysines on the αNTD (data not shown).

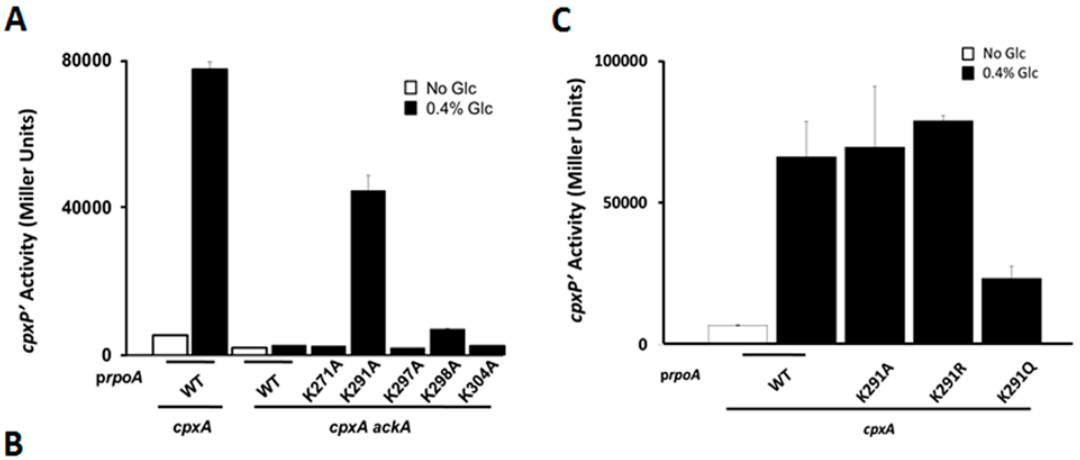
I also investigated whether Lys-291 could be responsible for the low *cpxP* response to 4% glucose in *cpxA* mutant cells (strain PAD348) whose Pta-AckA pathway remains intact. Indeed, cells that overexpressed the K291A variant yielded a stronger response to the presence of 4% glucose than did cells that overexpressed WT α (**Fig. 18B**). In contrast, Lys-291 was not required for the response to 0.4% glucose, as *cpxA* mutant cells that overexpressed either WT α or the K291A mutant responded at the same intensity (**Fig. 18C**). Therefore, whether caused by excessive glucose or by disruption of the Pta-AckA pathway, inhibition acts through Lys-291, perhaps due to its acetylation.

To determine if the requirement for Lys-291 could involve its acetylation, I took advantage of a genetic strategy commonly used in studies of eukaryotic protein acetylation (25). In this approach, the lysine (K) is converted to either a glutamine (Q) or an arginine (R). The KQ substitution mimics the neutral acetylated lysine, while the KR substitution mimics the positively charged unacetylated lysine. Consistent with the hypothesis that Lys-291 acetylation inhibits glucose-induced *cpxP* transcription, *cpxA* mutant cells overexpressing the K291Q mutant variant of α responded to glucose with less intensity than did cells that overexpressed WT α or its K291A or K291R mutant variants (**Fig. 18C**). This decrease in *cpxP* transcription, however, cannot be explained by a decrease in steady state levels of the protein, since I did not observe any difference in their steady-state level (data not shown)**.** These results lend support to the hypothesis the Lys-291 acetylation decreases the response to glucose by *cpxP.*

IS AN ACETYLTRANSFERASE REQUIRED FOR THE WEAK RESPONSE TO GLUCOSE?

In eukaryotes, protein acetylation is a reversible post-translational modification that is balanced by the action of a protein acetyltransferase that adds an acetyl group to the Nε amino group of a lysine, and a protein deacetylase that mediates the hydrolysis of the acetyl group. Therefore, I hypothesized that Lys-291 acetylation would require the action of an acetyltransferase and predicted that overexpression of this acetyltransferase should inhibit *cpxP* transcription, even when the Pta-AckA pathway is intact, while deletion of such an acetyltransferase should promote a strong response to glucose in the *ackA* mutant.

To identify an acetyltransferase that participates in Lys-291-dependent inhibition of *cpxP* transcription, I transformed WT cells with a set of multicopy plasmids; each plasmid expressed one of 22 known or predicted *E. coli* acetyltransferases (**Table 1**). I grew the resultant transformants in the presence of glucose and IPTG (to induce acetyltransferase expression), and measured β-galactosidase activity. Relative to the WT strain carrying the vector control plasmid, overexpression of 8 different acetyltransferases substantially inhibited the glucose response, while overexpression of only one increased the response (**Fig. 19A** and data not shown).



Growth Condition	No Glucose		0.4% Glucose		4.0% glucose	
rpoA variant	WT	K291A	WT	K291A	WT	K291A
Promoter activity /10³	8.20 (+/- 0.52)	6.05 (+/- 0.23)	79.00 (+/- 3.24)	66.29 (+/- 2.31)	35.80 (+/- 7.04)	48.05 (+/- 1.34)

Fig. 18. Lys-291 inhibits *cpxP* transcription. (A), *cpxP* lysogen of the *cpxA* mutant (strain PAD348) was transformed with plasmid pREII carrying the WT allele of *rpoA,* whereas the isogenic *cpxA ackA* mutant (strain AJW3827) was transformed with pREII carrying the WT allele of *rpoA* or lysine to alanine mutant derivatives of residues 271, 291, 297, 298, and 304. The resultant transformants were grown for 7.5 h at 37 °C with shaking in TB7 (*white bars*) or the same medium supplemented with 0.4% glucose (*black bars*). Cells were harvested at regular intervals and both *A*600 and β-galactosidase activity was measured. The *bars* indicate the means of triplicate independent cultures, and the *error bars* indicate the S.D. (B) β-galactosidase activity of *cpxP* lysogens of the *cpxA* mutant (strain PAD348) transformed with plasmid pREII carrying either the WT allele of *rpoA* (*white bars*), or the *rpoAK291A* mutant allele (*black bars*). The resultant transformants were grown at 37 °C with shaking in TB7. The same medium was supplemented with 0.4 or 4.0% glucose. Cells were harvested at regular intervals and both *A*600 and-galactosidase activity were measured. The fold-changes in -galactosidase activity from cells grown in the presence of glucose relative to those grown in the absence of glucose are reported in the histogram. The *bars* indicate the means of triplicate independent cultures, and the *error bars* indicate the S.D. Mean Miller units and S.D. from each condition are reported in the table. (C) *cpxP* lysogens of the *cpxA* mutant (strain PAD348) transformed with plasmid pREII carrying the WT allele of *rpoA*, lysine to alanine, lysine to arginine, and lysine to glutamine mutant derivatives of residue 291. Transformants were grown for 7.5 h at 37 °C with shaking in TB7 in the absence (*white bars*) or presence of 0.4% glucose (*black bars*). Cells were harvested at regular intervals and both *A*600 and -galactosidase activity was measured. The *bars* indicate the means of triplicate independent cultures, and the *error bars* indicate the S.D.

To test if one of these acetyltransferases was required for the weak response to glucose by the *ackA* mutant, I deleted each acetyltransferase from the *ackA* mutant (strain AJW3994) and screened for double mutants whose *cpxP* promoter could respond strongly to glucose. Only the *ackA wecD* double mutant exhibited strong *cpxP* transcription (**Fig. 19B**). However, this expression is unlikely to involve Lys-291 acetylation as it occurred even in the absence of glucose (**Fig. 19B inset**).

On the basis of these results, I concluded that multiple acetyltransferases could inhibit glucose-induced *cpxP* transcription, but that no single tested acetyltransferase may be required for this inhibition.

K291Q ALSO AFFECTS *cpxP* TRANSCRIPTION *IN VITRO*

The observed decrease in glucose-induced *cpxP* transcription, when I overexpressed several acetyltransferases, supports the hypothesis that protein acetylation can inhibit *cpxP* transcription. This hypothesis is also supported by the decrease in transcription when Lys-291 of α was substituted by a glutamine, which is thought to be a genetic mimic of an acetylated lysine. However, it remained unclear whether this potential effect of protein acetylation was a direct or indirect effect on the glucose-induced *cpxP* transcription. To distinguish between these two possibilities, I sought to first reconstitute *cpxP* transcription *in vitro* and then ask whether α K291Q had any effect in the *in vitro* transcription system.

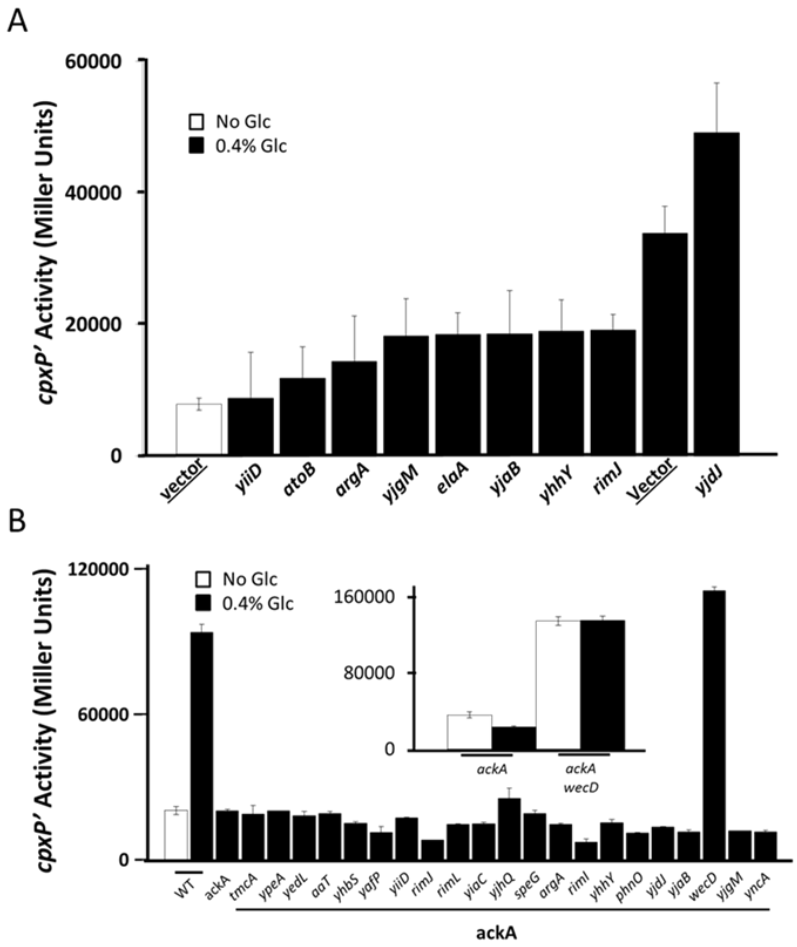


Fig. 19. Overexpression and deletion screens do not identify an acetyltransferase responsible for *ackA*-dependent inhibition

A) λ*cpxP* lysogens (strain PAD282) were transformed with the vector pCA24n or with pCA24n derivatives each carrying the ORF of a known or predicted acetyltransferase (52). Transformants carrying the vector control were grown for 7.5 hours at 37°C with shaking in TB7 (white bars) or the same medium supplemented with 0.4% glucose (black bars). Transformants carrying genes encoding known or predicted acetyltransferases were grown in medium supplemented with 0.4% glucose. Transformants were harvested at regular intervals and OD_{600} and β-galactosidase activity were measured. Only the values at the last time point are shown. The bars indicate the means of two experiments, each conducted with duplicate independent cultures. The error bars indicate the standard deviations.

B) λ*cpxP* lysogens of an *ackA* mutant (strain AJW3994) and a set of double mutants, in which the *ackA* mutation was combined with deletions of known or predicted acetyltransferases (strains AJW4303-4323). All mutants were grown in TB7 supplemented with 0.4% glucose (black bars). λ*cpxP* lysogens of the WT (strain PAD282) were grown in TB7 in the absence (white bars) or presence of 0.4% glucose (black bars). Cells were harvested at regular intervals and OD_{600} and β-galactosidase activity were measured. Only the values at the last time point are shown. The bars indicate the means two experiments, each conducted with duplicate independent cultures. The error bars indicate the standard deviations.

(Inset) λ*cpxP* lysogens of the *ackA wecD* double mutant grown in TB7 in the absence (open bars) or presence of 0.4% glucose (close bars). Cells were harvested at regular intervals and OD_{600} and β-galactosidase activity were measured. Only the values at the last time point are shown. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.

To establish the *in vitro* transcription system, I combined purified WT RNA polymerase containing σ^{70} ($E\sigma^{70}$) with purified WT or D51A CpxR, purified plasmid *p770* carrying a DNA fragment that includes the *cpxP* promoter (**Table 1**) and AcP in an *in vitro* transcription buffer. The reactions were incubated at 30°C for 15 minutes and transcription was visualized by incorporation of [^{32}P]-labeled UTP into the transcripts. To the extent that I tested, *in vitro* transcription of *cpxP* seems to have the same requirements as its *in vivo* transcription: AcP (**Fig. 20**), CpxR (**Fig. 21**), and Asp-51 (**Fig. 22**) the phosphorylation site of CpxR. These results support the use of this system to test whether the effect of α K291Q on *cpxP* transcription is a direct or indirect effect. To that end, I combined purified RNAP that contained αK291Q with purified WT CpxR, purified *p770* carrying *cpxP* and AcP in an *in vitro* transcription buffer. The reactions were incubated at 30°C for 15 minutes and transcription was visualized by incorporation of [^{32}P]-labeled UTP into the transcripts. When compared to WT, RNAP that contained αK291Q was defective for *cpxP* transcription. This defect in transcription, however, does not seem to affect the overall function of RNAP as *rna1* transcription, which does not require the αCTD, seemed unaffected by the mutation at Lys-291 (**Fig. 23**).

The αCTD is an important transcription regulator and it does so by directly interacting with that UP element, an AT-rich region found in some but not all promoters, and/or by directly interacting with transcription factors; in either case, the αCTD is thought to aid in the recruitment of the holoenzyme to the promoter region. Therefore, if αK291Q could somehow lead to a misfolded domain, this mutation

could affect virtually any promoter that requires the αCTD for proper regulation. However, this mutation could have more specific and targeted effects; for example, if the αK291Q does not cause any general folding defect.

To distinguish between these two possibilities, I took advantage of the large amount of molecular information available on the interaction between αCTD and the ribosomal RNA promoter *rrnB.* Proper transcriptional regulation of *rrnB* requires the αCTD, but there is no evidence that Lys-291 plays any role in this regulation. Therefore, I predicted that if the defect in *in vitro cpxP* transcription, as seen with αK291Q, was due to improper CTD folding, then it also should affect *rrnB* transcription *in vitro*. However, if αK291Q was able to properly fold its CTD, then *rrnB* transcription should not be affected, as αK291 is not involved in *rrnB* transcription. Consistent with the idea that αK291Q is capable of properly folding its CTD, there was no visible difference in *rrnB in vitro* transcription between reactions with WT RNAP and those with αK291Q RNAP.

On the basis of these results, I conclude that substitution of glutamine for lysine at position 291 of α has a direct effect on *cpxP* transcription. Additionally, this defect seems to be specific to *cpxP*, as this substitution does not affect transcription of the *rrnB* promoter, which has been shown to require the αCTD, but not Lys-291. Since glutamine is considered a mimic of acetylated lysine and since Lys-291 is acetylated in the *ackA* mutant, I propose that acetylation of Lys-291 is responsible for the weak *cpxP* transcription observed in the *ackA* mutant.

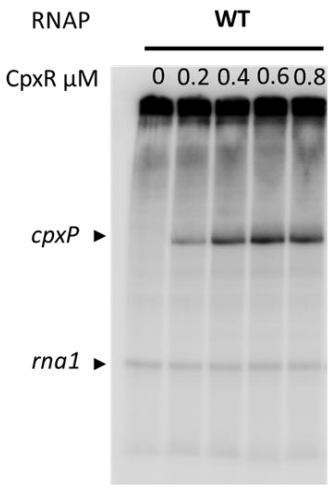


Fig. 20. CpxR is required for *cpxP* transcription *in vitro*. *in vitro* transcription of *cpxP* with increasing concentration of His6-CpxR. The reaction contained 10 nM $E\sigma^{70}$ and 10 mM AcP. Each reaction was incubated for 15 minutes at 30°C and transcripts were visualized by incorporation of ^{32}P-labeled UTP. *rna1* transcription serves as transcription standard control.

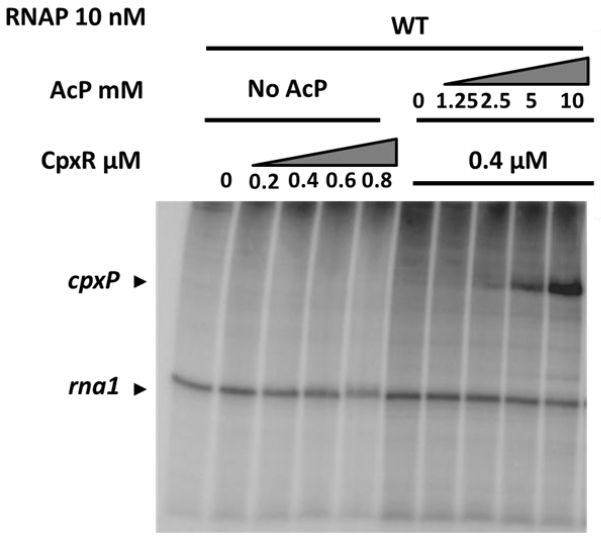


Fig. 21. AcP is required for *in vitro cpxP* transcription. *in vitro* transcription of *cpxP* with increasing concentration of His6-CpxR with no AcP (**left**), or increasing concentration of AcP with (**right**). Each reaction contained 10 nM $E\sigma^{70}$ and was incubated for 15 minutes at 30°C. Transcripts were visualized by incorporation of ^{32}P-labeled UTP. *rna1* transcription serves as transcription standard control.

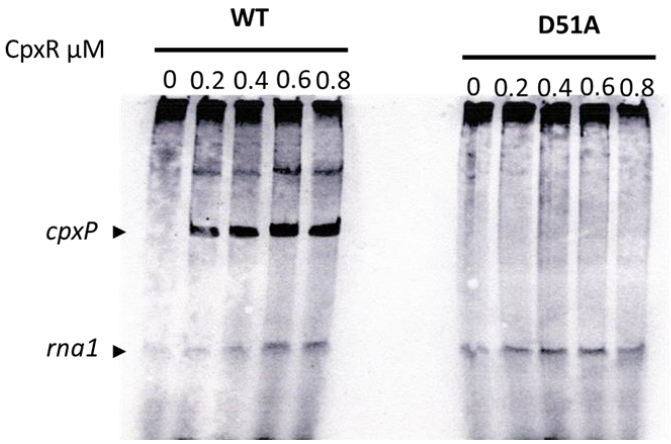


Fig. 22. Asp-51 of CpxR is required for *in vitro cpxP* transcription. *in vitro* transcription of *cpxP* with increasing concentration of His6-CpxR (**left**), or increasing concentration His6-CpxRD51A (**right**). Each reaction contained 10 nM $E\sigma^{70.}$ and 10 mM AcP. The reaction were incubated for 15 minutes at 30°C. Transcripts were visualized by incorporation of ^{35}S-labeled UTP. *rna1* transcription serves as transcription standard control.

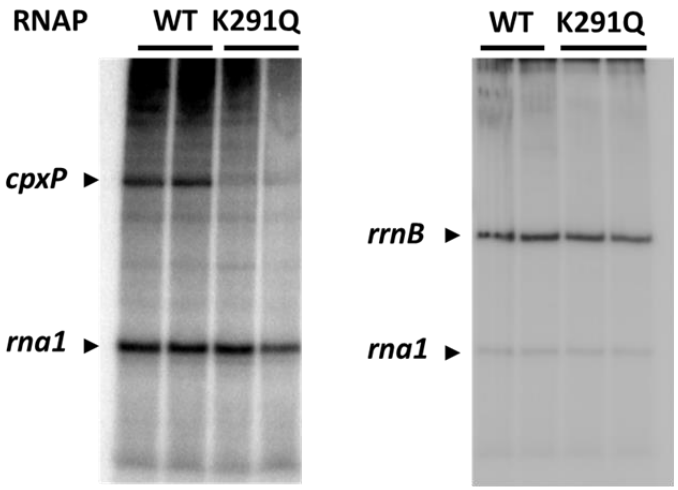


Fig. 23 Lys-291 of αCTD is required for *in vitro cpxP* transcription, but not *rrnB*. (left) *in vitro* transcription of *cpxP* with either WT $E\sigma^{70}$ or $E\sigma^{70}$ that contained αK291Q at 10 nM. Each reaction also contained 10 mM AcP and t 10 mM and 0.2 μM His6-CpxR. (**right**) *in vitro* transcription of *rrnB* with either WT $E\sigma^{70}$ or $E\sigma^{70}$ that contained αK291Q at 10 nM. Each reaction also contained 10 mM AcP and t 10 mM and 0.2 μM His6-CpxR. The reaction were incubated for 15 minutes at 30°C. Transcripts were visualized by incorporation of ^{32}P-labeled UTP. *rna1* transcription serves as transcription standard control.

CONCLUSION

I previously demonstrated that AcP could function as a phosphoryl donor, both *in vitro* and *in vivo*, to the RR CpxR. However, I hypothesized that this transfer of phosphoryl group was not the only regulatory mechanism involved in the CpxA-independent, glucose-induced *cpxP* transcription. I had come to this hypothesis because *ackA* mutants failed to induce high *cpxP* transcription in response to the addition of glucose, even though CpxR was phosphorylated under these conditions. Thus, the goal of this chapter was to test the hypothesis that, in addition to CpxR phosphorylation, additional a regulatory mechanism(s) control(s) *cpxP* transcription. The data presented here dismiss catabolite repression and implicate protein acetylation as that additional regulator, perhaps via Lys-291 of α.

With the help of Dr. Antelmann and her group, we determined that RNAP acetylation was enriched when WT and *ackA* mutant cells were grown in TB7 supplemented with 0.4% glucose relative to unsupplemented TB7. The acetylated lysines from both WT and *ackA* mutant samples were distributed throughout the RNAP subunits that we analyzed. However, the distribution was different in the two different strains. In each strain, some of the acetylations were located near sites that could impact RNAP function (i.e. the DNA binding patch of the αCTD and near the secondary channel between β and β'). This lends credence to the idea that acetylation could regulate activity. I tested this concept by converting each of the acetylated lysines of α to an alanine and learned that Lys-291 was involved in the weak response to 0.4% glucose by the *ackA* mutant and in the response to 4% glucose by

the WT parent. Furthermore, conversion of lysine 291 to the acetylated lysine mimic glutamine (K291Q) inhibited *cpxP* transcription both *in vivo* and *in vitro*. Although the finding that αK291 is involved in dampening *cpxP* transcription when WT cells are exposed to 4% glucose leads me to hypothesize that Lys-291 involvement in the 4% response is due to its acetylation, this hypothesis remains to be tested and at this point we do not have mass spectrometry evidence that Lys-291 becomes acetylated in the presence of 4% glucose.

If the K291Q substitution is a true mimic for acetylation of that residue, then the K291Q inhibition of *cpxP* transcription is evidence that RNAP acetylation directly impacts *cpxP* transcription, dampening it when compared to unacetylated RNAP. The effect of Lys-291 acetylation, however, does not seem to affect all αCTD-dependent promoters, as the well characterized *rrnB* promoter was unaffected by αK291Q, even though its transcription requires the αCTD.

The mechanism by which K291 becomes acetylated in the *ackA* mutant background remains unknown. Overexpression of several known and putative acetyltransferases inhibited *cpxP* transcription in cells with an intact Pta-AckA pathway, yet deletion of no single acetyltransferase was sufficient to relieve the inhibition of *cpxP* transcription observed in *ackA* mutants. It is possible that multiple acetyltransferases could acetylate K291. Alternatively, acetylation of this residue might occur in a manner that does not require an acetyltransferase. This issue will be address in the next chapter.

Although Lys-291 of α was the only tested lysine involved in *cpxP* transcription, β and β' are also widely acetylated. This raises the possibility that some of these acetylation events could also affect transcription. Additionally, α, β and β' were also found to be acetylated even when the Pta-AckA pathway was intact, albeit on different residues, as long as the cells were grown in the presence of glucose. This raises the question: could any of these acetylation events of RNAP from WT cells also affect transcription from *cpxP* or from other promoters? This question will be addressed in the next chapter.

CHAPTER FIVE
RESULTS

ACETYLPHOSPHATE AS AN ACETYL DONOR

I previously showed evidence that transcription of the *cpxP* promoter can be regulated both by the canonical CpxA-dependent phosphorylation of CpxR and by a CpxA-independent mechanism, via an AcP-dependent phosphorylation. I also provided evidence to support the hypothesis that this AcP-dependent behavior is modulated by acetylation of Lys-291 on the αCTD of RNAP. Acetylation of α Lys-291 was detected when *ackA* mutant cells were grown in the presence of 0.4% glucose. Thus, I proposed that RNAP acetylation is a novel regulatory mechanism of the *cpxP* promoter. However, we also detected RNAP acetylation in WT cells that contained an intact Pta-AckA pathway. These observations raise the possibility that some of these WT acetylation events affect RNAP function. In this chapter, I will ask if glucose-induced *cpxP* transcription can be affected by protein acetylation when the cells contain an intact Pta-AckA pathway.

cpxP TRANSCRIPTION RESPONDS TO ALTERATIONS IN THE ACETYLOME

To test the hypothesis protein acetylation affects *cpxP* transcription in WT cells, I exposed the *cpxA* mutant (strain PAD348) to increasing concentrations of nicotinamide, an inhibitor of NAD^+-dependent sirtuin-like protein deacetylases. If protein acetylation influences *cpxP* transcription in WT cells, I would expect that this transcription would be affected by the addition of nicotinamide to the media. In support of the hypothesis that *cpxP* transcription is affected by protein acetylation, I observed a CpxA- and glucose-independent increase in *cpxP* transcription that increased as the concentration of nicotinamide increased, achieving more than a 4-fold induction at 50 mM nicotinamide, even in the absence of glucose (**Fig. 24A**).

Since nicotinamide can induce CpxA-independent *cpxP* transcription and glucose can induce protein acetylation, I hypothesized that the glucose-induced *cpxP* transcription could involve protein acetylation. To test this hypothesis, I overexpressed the NAD^+-dependent deacetylase CobB (pCA24n-*cobB*; Table 2) in WT cells (strain PAD282) grown in the presence of 0.4% glucose. If the *cpxP* response to glucose requires protein acetylation, then cells that overexpress CobB should respond less strongly to glucose when compared to vector control. Indeed, overexpression of CobB reduced the 6-fold glucose-dependent induction to a more modest 3-fold induction (**Fig. 24B**).

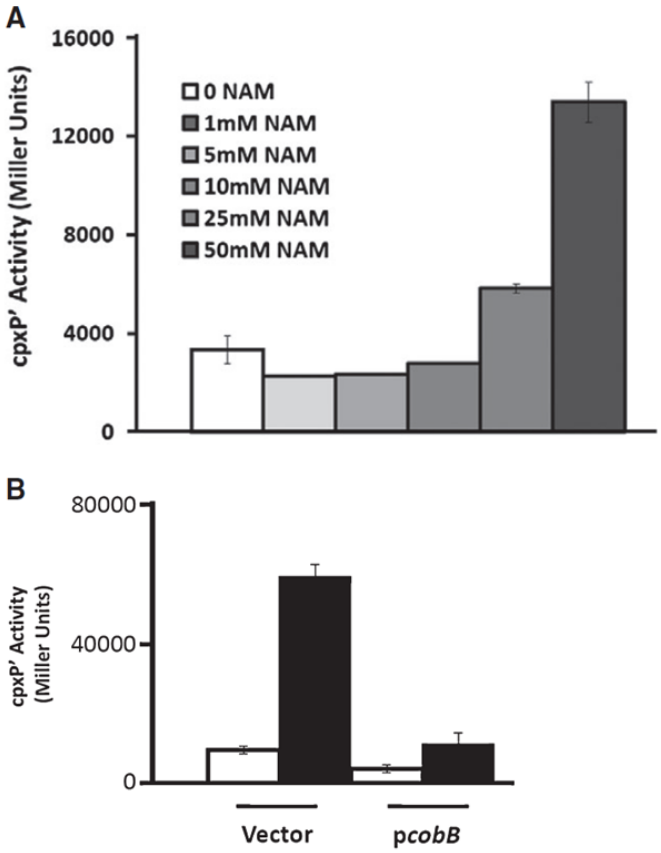


Fig. 24. Nicotinamide induces CpxA-independent *cpxP* transcription, whilst CobB overexpression inhibits it.
A) A λ*cpxP* lysogen of the *cpxA* mutant (PAD348) was grown at 37°C with shaking in buffered TB (open bars) or the same medium supplemented with increasing concentrations of nicotinamide (NAM, closed bars). Cells were harvested after 7.5 hours incubation and β-galactosidase activity was measured. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.
B) A λ*cpxP* lysogen of the wild-type strain (PAD348) was transformed with the vector pCA24n or with pCA24n derivative carrying the *cobB* ORF. Transformants were grown at 37°C with shaking in buffered TB supplemented with 5 µM IPTG (open bars) or the same medium supplemented with both 0.4% glucose and 5 µM IPTG (closed bars). Cells were harvested after 7.5 hours incubation and β-galactosidase activity was measured. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.

To determine whether the above mentioned treatments could influence the overall protein acetylation pattern, I used a cocktail of two polyclonal anti-acetylated-lysine antibodies to detect overall protein acetylation via Western immunoblot analysis of whole cell lysates (**Fig. 15**). When WT cells (strain PAD282) were grown in the presence of either nicotinamide or glucose, the acetylome changed relative to cells grown without treatment. Glucose generally caused the number and/or intensity of acetylated bands to increase, as reported previously in chapter 3. In contrast, the deacetylase inhibitor nicotinamide induced more subtle changes. These subtle changes in the acetylation pattern correlated with a weaker response by the *cpxP* promoter to the addition of nicotinamide when compared to its response to glucose. CobB overexpression in cells exposed to glucose yielded a strong effect on the acetylation pattern. This acetylome closely resembled that of cells grown in the absence of glucose or nicotinamide. Thus, the conditions that influence *cpxP* transcription correlate with a change in the *E. coli* acetylome.

GLUCOSE-INDUCED *cpxP* TRANSCRIPTION REQUIRES A LYSINE ACETYLTRANSFERASE

In *S. enterica*, the NAD^+-dependent deacetylase CobB works in conjunction with the GCN5-like acetyltransferase Pat to regulate the activity of the enzyme acetyl-CoA synthetase (124, 125). The finding that CobB overexpression could inhibit *cpxP* transcription led us to hypothesize that glucose-induced *cpxP* transcription could be regulated by the *E. coli* Pat homolog, YfiQ.

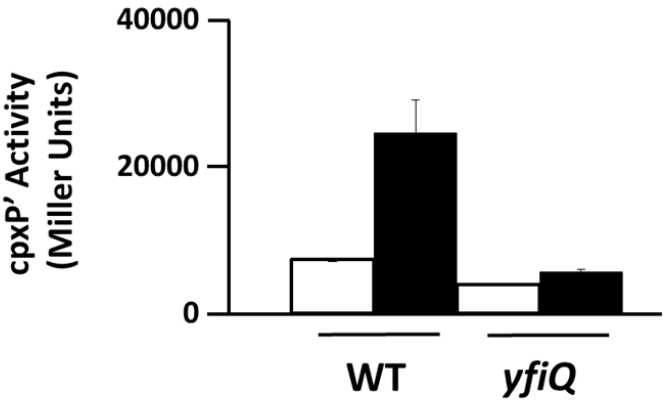


Fig. 25. The carbon response requires a lysine acetyltransferase.
λ*cpxP* lysogens of the *yfiQ* mutant (strain AJW3143) and its wild-type parent (PAD 282) were grown at 37°C with shaking in buffered TB (open bars) or the same medium supplemented with 0.4% glucose (closed bars). Cells were harvested after 7.5 hours incubation and β-galactosidase activity was measured. The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.

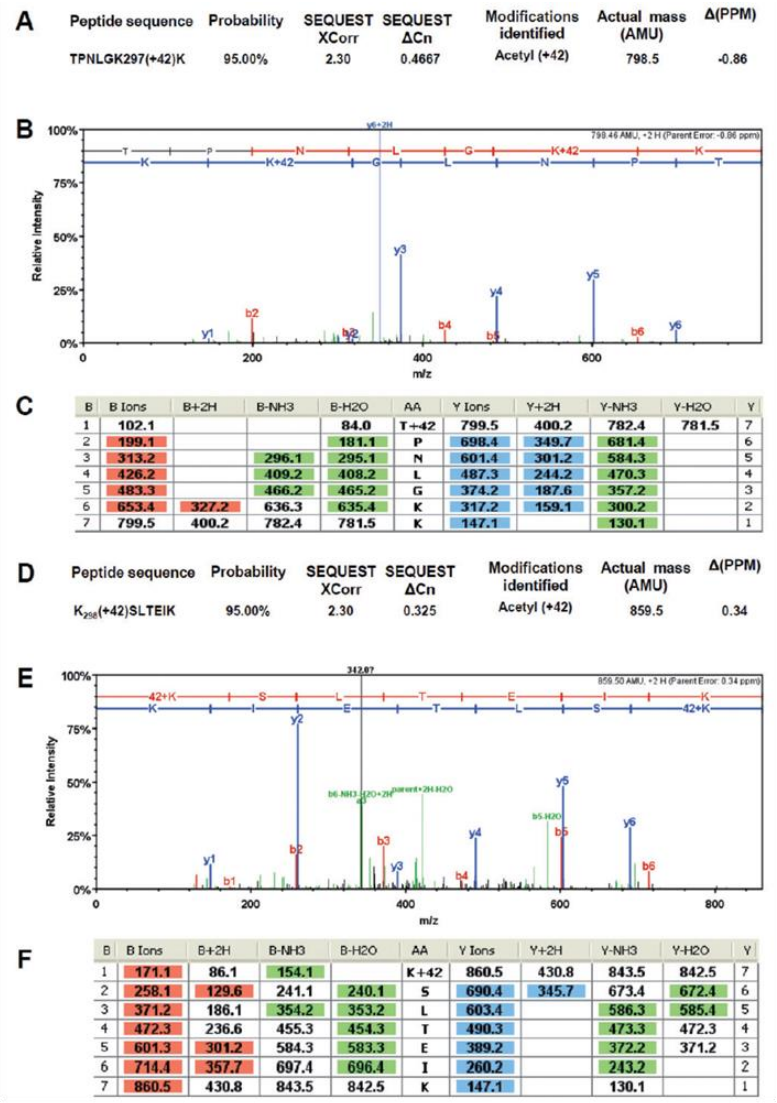
To test the hypothesis that YfiQ regulates glucose-induced *cpxP* transcription, I compared the glucose response of a mutant that lacks YfiQ (strain AJW3143; Table 1) to that of its WT parent (strain PAD282). In contrast to its WT parent (approximately 3.5-fold induction), the *yfiQ* mutant responded poorly to glucose (approximately 1.5-fold induction) (**Fig. 25**). The requirement for YfiQ, however, did not extend to CpxA-dependent *cpxP* transcription, as surface-induced *cpxP* transcription was equivalent in both the *yfiQ* mutant and its WT parent (data not shown). Thus, the requirement for YfiQ is specific for the response to additional carbon. Therefore, I proposed that YfiQ and CobB catalyze the reversible acetylation of a protein, and that acetylation is required for glucose-induced *cpxP* transcription.

YfiQ and CobB are known to catalyze the reversible acetylation of acetyl-CoA synthetase (Acs) (124, 125). To test if Acs was involved in glucose-induced *cpxP* transcription, I deleted its gene; however, I did not observe any effect on *cpxP* transcription (data not shown). This result was not surprising since *acs* transcription is inhibited by catabolite repression (73). I therefore sought the *cpxP*-relevant target of YfiQ and CobB elsewhere.

YfiQ IS REQUIRED FOR *IN VIVO* ACETYLATION OF MULTIPLE LYSINES ON RNAP

The finding that protein acetylation was required for glucose-induced *cpxP* transcription, that RNAP is acetylated when WT cells is grown in the presence of glucose, and that acetylation of lys-291 of α seems to inhibit glucose-induced *cpxP*

transcription in the *ackA* mutant, led me to begin my search for the acetylation target(s) of YfiQ by asking whether glucose-induced RNAP acetylation required YfiQ. To that end, I grew WT and *yfiQ* mutant cells (strain AJW3143) in the absence or presence of 0.4% glucose, isolated the RNAP subunits by immunoprecipitation and subjected them to MS analysis. Using linear trap quadrupole (LTQ) Orbitrap mass spectrometry (MS), Dr. Haike Antelmann and her group first identified the acetylation sites on RNAP purified from WT cells. They mapped 15 glucose-dependent acetylation sites to β (**Table 4**), 11 acetylation sites to β' (**Table 4**), and 2 acetylation sites to α (**Fig. 26)**. None of these sites were acetylated when WT cells were grown in the absence of glucose. Of the 15 different acetylation sites detected from β, they detected 14 in at least two biological replicates. Of the 11 acetylation sites detected from β', they reproducibly detected 8 (data not shown). Finally, α peptides containing acetylated Lys-297 or acetylated Lys-298 were detected in multiple biological replicates. Ten of the fifteen glucose-dependent β acetylations, ten of the eleven glucose-dependent β' acetylations, and one of the two glucose-dependent α acetylations (Lys-298) required YfiQ (**Table 4).** Dr. Antelmann's group detected no acetylated peptides from *yfiQ* mutant cells grown in the absence of glucose, which parallels the results with RNAP isolated from WT cells grown in the absence of glucose. Thus, I conclude that YfiQ is required for most of the glucose-induced acetylations of RNAP *in vivo*.



B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	102.1			84.0	T+42	799.5	400.2	782.4	781.5	7
2	199.1			181.1	P	698.4	349.7	681.4		6
3	313.2		296.1	295.1	N	601.4	301.2	584.3		5
4	426.2		409.2	408.2	L	487.3	244.2	470.3		4
5	483.3		466.2	465.2	G	374.2	187.6	357.2		3
6	653.4	327.2	636.3	635.4	K	317.2	159.1	300.2		2
7	799.5	400.2	782.4	781.5	K	147.1		130.1		1

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	171.1	86.1	154.1		K+42	860.5	430.8	843.5	842.5	7
2	258.1	129.6	241.1	240.1	S	690.4	345.7	673.4	672.4	6
3	371.2	186.1	354.2	353.2	L	603.4		586.3	585.4	5
4	472.3	236.6	455.3	454.3	T	490.3		473.3	472.3	4
5	601.3	301.2	584.3	583.3	E	389.2		372.2	371.2	3
6	714.4	357.7	697.4	696.4	I	260.2		243.2		2
7	860.5	430.8	843.5	842.5	K	147.1		130.1		1

Fig. 26. The α subunit of RNAP is acetylated on K297 and K298.
Immunoprecipitated subunits of RNAP were separated by SDS-PAGE. The α bands were excised and tryptically digested as described previously (Chi *et al.*, 2010). The resulting peptides were analyzed in a LTQ OrbitrapXL™ mass spectrometer as described in the Materials and Methods. The double charged acetyllysine-modified peptides TPNLGK$_{297}$(+42)K and K$_{298}$(+42)SLTEIK were detected as mass peaks of m/z=400,237 and m/z=430,7582, respectively, in the digested α sample. A and D) The Xcorr and ΔCn scores, the actual peptide masses and mass deviations of the acetylated peptides. B and D) The corresponding CID MS/MS spectra. C and E) Complete b and y fragment ion series for these peptides.

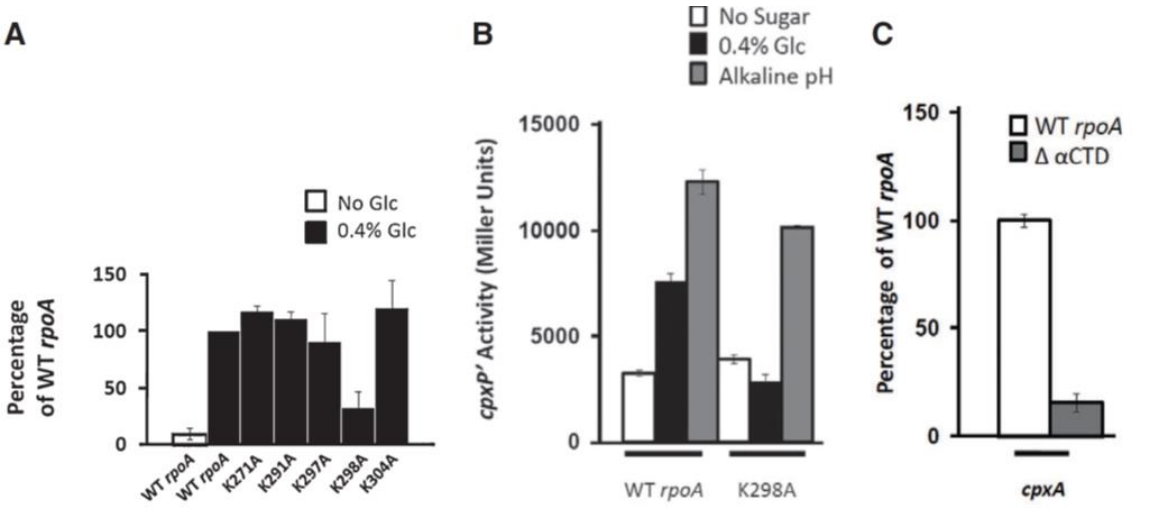


Fig. 27. Figure 8. Glucose-induced *cpxP* transcription requires K298 of αCTD.

A) β-galactosidase activity of a λ*cpxP* lysogen of the *cpxA* mutant (strain PAD348) transformed with plasmid pREII carrying the wild-type allele of *rpoA,* or lysine-to-alanine mutant derivatives of residues 271, 291, 297, 298 and 304. The resultant transformants were grown at 37°C with shaking in buffered TB (open bars) or the same medium supplemented with 0.4% glucose (closed bars). The wild-type response to glucose was set at 100% and the response observed by each of the mutant derivatives was expressed as a percentage of the activity exhibited by wild-type when exposed to 0.4% glucose. The bars indicate the mean of 5 independent experiments carried out with three independent cultures, and the error bars indicate the standard deviations.

B) β-galactosidase activity of λ*cpxP* lysogens of the *cpxA* (PAD348) transformed with plasmid pREII carrying the either wild-type *rpoA* allele or the K298A mutant allele. Transformants were grown at 37°C with shaking in TB buffered at pH 7.0 (open bars), the same medium supplemented with 0.4% glucose (closed bars), or TB buffered at pH 7.8 (gray bars). The bars indicate the means of triplicate independent cultures, and the error bars indicate the standard deviations.

C) β-galactosidase activity of λ*cpxP* lysogens of the *cpxA* mutant (strain PAD348) transformed with plasmid pREII carrying the WT allele of *rpoA* (white bar), or an *rpoA* mutant allele lacking the CTD (gray bar). Transformants were grown at 37°C with shaking in buffered TB medium supplemented with 0.4% glucose. *cpxP* transcription of the wild-type response to glucose was set at 100% and the response observed by the Δ αCTD mutant derivative was expressed as a percentage of the activity exhibited by the wild-type when exposed to 0.4 % glucose. The bars indicate the mean of 5 independent experiments carried out with three independent cultures, and the error bars indicate the standard deviations.

GLUCOSE-INDUCED TRANSCRIPTION REQUIRES LYS-298 OF THE αCTD

That acetylation of several lysines on RNAP is both glucose and YfiQ-dependent led me to hypothesize that at least one of these residues could be required for glucose-induced *cpxP* transcription. To test this hypothesis, I focused my attention on α. I chose α due to its smaller size relative to β and β', to our finding that acetylation of only one residue (Lys-298) was both glucose and YfiQ-dependent and to the finding that, under different conditions, an acetylatable α residue (Lys-291) influences *cpxP* transcription. To test whether Lys-298 or any of the other four lysines on the αCTD were required for glucose-induced *cpxP* transcription, I introduced plasmids that carry either the WT *rpoA* allele or lysine-to-alanine mutant derivatives into the *cpxA* mutant (strain PAD348), which carries the WT *rpoA* gene at its native location on the chromosome. I grew the resultant transformants in the absence or presence of 0.4% glucose and compared the β-galactosidase activity of cells expressing each lysine-to-alanine substitution to that of cells expressing WT α. Of the five mutants tested, only K298A exerted a defect on glucose-induced *cpxP* transcription (**Fig. 27A**).

To ensure that the K298A mutant retained function, I tested if a CpxA-dependent stimulus (i.e., exposure to alkaline pH) could induce *cpxP* transcription in cells WT for CpxA (strain PAD282). Indeed, cells expressing the K298A mutant activated *cpxP* transcription when grown in TB buffered at pH 7.8, but not in TB buffered at pH 7.0 and supplemented with glucose. In contrast, cells expressing WT *rpoA* activated *cpxP* transcription under both conditions (**Fig. 27B**).

I further tested whether the loss of glucose-induced *cpxP* transcription observed with the K298A mutant was due to a dominant-negative effect by measuring β-galactosidase activity of cells that carried a mutant α lacking its CTD. Into the *cpxA* mutant (PAD348), I introduced plasmids that carry either the WT *rpoA* allele or a mutant derivative with the CTD and the linker region deleted. I grew resultant transformants in the absence or presence of glucose and measured β-galactosidase activity. Like the K298A mutant, the αCTD deletion mutant exhibited a defect in glucose-induced *cpxP* transcription (**Fig. 27C**). I interpreted these results to mean that K298A allele encodes a functional form of αCTD and that Lys-298 is required for glucose-induced *cpxP* transcription, but not for CpxA-induced transcription, perhaps because Lys-298 needs to be acetylated under these conditions.

YfiQ DOES NOT ACETYLATE RNAP *IN VITRO*

The finding that YfiQ and Lys-298 of α were required for glucose-induced *cpxP* transcription and that YfiQ was required for Lys-298 acetylation *in vivo*, led me to hypothesize that YfiQ directly acetylated Lys-298. To test this hypothesis I attempted to acetylate α by incubating purified His_6-YfiQ, [^{14}C]-labeled AcCoA, and Eσ^{70} into the *in vitro* transcription buffer for 1 hour at 37°C. However, in spite of my efforts, I was unable to obtain any evidence that YfiQ acetylated α, or any other RNAP subunit, under my experimental conditions. This raises that possibility that RNAP is not directly acetylated by YfiQ.

AcP AS AN ACETYL DONOR

My inability to acetylate RNAP *in vitro* using YfiQ as the acetyltransferase and AcCoA as the acetyl donor, led me to consider the possibility that YfiQ does not directly acetylate RNAP and that the observed effect on *in vivo* RNAP acetylation, when YfiQ was deleted, was an indirect effect.

Several factors led me to consider the possibility that AcP itself was the acetyl donor to RNAP. (i) Western immunoblot analysis conducted by Bozena Zemaitaitis and quantitative mass spectrometry performed by Birgit Schilling in Bradford Gibson's lab (Buck Institute of Aging) demonstrated that the accumulation of intracellular AcP correlates with a dramatic increase in total protein acetylation. (ii) Linda Hu (from our lab) possesses evidence that the central metabolite protein LpdA can be acetylated *in vitro* in an AcP-dependent and acetyltransferase-independent manner. (iii) Misty Kuhn and Michael Scholle from the labs of Wayne Anderson and Milan Mrksich (Northwestern University) possess evidence that AcP can function as a direct acetyl donor to linear peptides containing a lysine.

To test if AcP could function as an acetyl donor to RNAP, I incubated $E\sigma^{70}$ in *in vitro* transcription buffer with increasing concentration of AcP at 30°C for 1 hour and detected RNAP acetylation via Western immunoblot analysis. Consistent with the hypothesis that AcP can function as an acetyl donor to RNAP, I detected an increase in the acetylation of β and/or β' as the concentration of AcP in the reaction increased. This result could help explain a conundrum that *in vivo cpxP* transcription

requires an acetylation event; however, *in vitro* transcription occurs in the absence of an acetyltransferase or AcCoA (**Fig. 28**).

CONCLUSION

There are over 300 known posttranslational modifications (143). Protein phosphorylation is likely the most studied of all these posttranslational modifications. However, in the recent years, protein acetylation has been gaining increased attention in all domains of life. Based on global proteomics studies [(68, 139, 155, 157) and Schilling, Gibson and Wolfe, unpublished], protein acetylation is likely to affect many proteins that play important roles in *E. coli* and *S. enterica* physiology. In *E. coli*, some of these proteins are the subunits of RNAP.

In chapter 4, I provided evidence that supports the hypothesis that acetylation of Lys-291 of the α subunit of RNAP inhibits glucose-induced *cxpP* transcription in *ackA* mutants. In this chapter, I extended the effect of protein acetylation and perhaps of RNAP acetylation to include glucose-induced *cpxP* transcription from cells that contain a WT Pta-AckA pathway.

Mass spectrometry analysis conducted by Dr. Haike Antelmann's group demonstrated that glucose-induced acetylation of several lysines on α, β and β' required the acetyltransferase YfiQ. Among the sites affected by deletion of *yfiQ* is Lys-298 of α and conversion of Lys-298 to an alanine had profound impact in glucose-induced *cpxP* transcription. YfiQ is also required for glucose-induced *cpxP* transcription. Thus, together these data support the hypothesis that glucose-induced *cpxP*

transcription is dependent on the YfiQ-dependent acetylation of α Lys-298. Lys-298 is important also for transcription of the rRNA promoter *rrnB.* At the *rrnB* promoter Lys-298 makes direct contact with the DNA backbone. Thus, acetylation of this residue could potentially have broad impact in *E. coli* physiology.

Despite being required for acetylation of approximately 70% of the lysines acetylated by glucose, including Lys-298, I was unable to obtain *in vitro* evidence that YfiQ directly acetylates any of those lysines. This raises the possibility that the YfiQ effect on RNAP acetylation is indirect. In fact, my data indicate that AcP is more likely to be directly responsible for RNAP acetylation than YfiQ, as I was able to detect, by Western immunoblot analysis, an increase in acetylation of β and/or β' when RNAP was incubated with increasing concentration of AcP. Additional analysis, via mass spectrometry, is required to determine if other RNAP subunits are also acetylated by AcP and if so their identity.

AcP had previously been proposed to play an important role in *E. coli* physiology due to its ability to donate its phosphoryl groups to some response regulators *in vitro.* In chapter 3, I demonstrated that this transfer of phosphoryl group from AcP to RRs also occurs *in vivo* to the RR CpxR. However, based on evidence presented in this chapter, this AcP-dependent phosphorylation seems to be modulated by another AcP-dependent posttranslational protein modification, the acetylation of RNAP. This acetylation sees to function in some sort of balancing acting, modulating the strength of the AcP-dependent *cpxP* transcription.

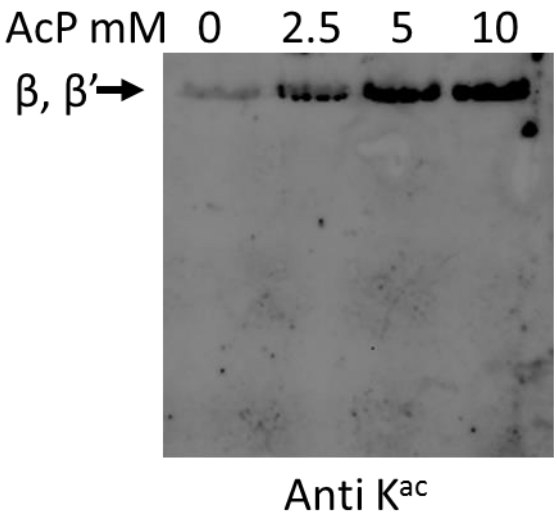


Fig. 28. *in vitro* acetylation with AcP. Purified $E\sigma^{70}$ was incubated with increasing concentration of AcP for 1 hour at 30°C. After incubation the proteins were resolved by SDS-PAGE and transferred to a membrane. Proteins acetylation was detected using a cocktail of anti-acetylated lysine antibodies.

The *E. coli* genome is predicted to encode over 20 known or putative Gcn5-like acetyltransferases. Of those, YfiQ is likely the best characterized, and is the only one to be demonstrated to possess Nε lysine acetylation capabilities. However, global mass spectrometry analysis performed by Birgit Schilling and Bradford Gibson detected only a 10-20% reduction in the total number of proteins acetylated when *yfiQ* was deleted from *E. coli* cells grown in the presence of 0.4% glucose. In contrast, deletion of *ackA* doubled the number of acetylated proteins, under the same conditions, and deletion of both *pta* and *ackA* decreased the number of total acetylated proteins by 50% in the absence of glucose. Thus, I concluded that, under the tested condition, AcP has a more important role in protein acetylation than the acetyltransferase YfiQ. This effect is likely to be a combination of direct and indirect acetylation as transcription of the putative acetyltransferase YafP is increased in cells that accumulate AcP and AcP can serve as the acetyl donor for at least two proteins *in vitro,* LpdA and RNAP.

CHAPTER SIX

DISCUSSION

OVERALL SUMMARY

As part of the long-term effort by the Wolfe laboratory to map the impact of AcP on the network of *E. coli* 2CST pathways, I discovered that AcP has a significant effect on CpxR-dependent transcriptional regulation of *cpxP*. The evidence I presented here supports a model in which AcP plays a pivotal role in the transcription regulation of *cpxP* via both phosphorylation of the RR CpxR and acetylation of RNAP. These two PTMs seem to work together to fine tune *cpxP* transcription as the intensity of the AcP-dependent *cpxP* transcription seems to be affected by acetylation of different lysine residues on the αCTD. While the hypothesis that AcP can phosphorylate and therefore control the activity of multiple response regulators has been proposed before (90, 91), the notion that AcP can also serve as an acetyl donor is fairly novel. This newly identified role for AcP has the potential to open several new lines of investigation. Some questions come to mind: What is the overall impact of this AcP-dependent acetylation? It seems evident that RNAP acetylation has the potential to affect multiple aspects of *E. coli* physiology. How many more proteins are

acetylated in the same manner? Some recent data from the Wolfe laboratory demonstrate that AcP has a large impact on the overall protein acetylation status of *E. coli*, as demonstrated by Western immunoblot analysis and mass spectrometry analysis. How prevalent is AcP-dependent acetylation when compared to AcCoA-dependent acetylation? Does AcP-dependent acetylation occur in other organisms?

The Wolfe laboratory has started to address some of these questions and, based on some of the data obtained so far, it seems like AcP has a broader impact in *E. coli* physiology than previously appreciated.

SIGNAL INTEGRATION

On the basis of my observations and previously published reports, I propose that the CpxAR pathway integrates multiple signals at three distinct levels. (i) In the periplasm, CpxP integrates stimuli predicted to cause misfolding of envelope proteins [for reviews, see references (31, 32, 106, 115)]. (ii) At the cytoplasmic membrane, the periplasmic domain of CpxA integrates information concerning the status of the periplasmic chaperone CpxP with signals that depend on the outer membrane lipoprotein NlpE and with stimuli that depend on neither CpxP nor NlpE (11, 30, 98, 107, 110, 151). (iii) Finally, in the cytoplasm, CpxR integrates the above mentioned CpxA-dependent signals with those that are independent of CpxA (e.g., entry into stationary phase and exposure to excess carbon (23, 149).

It has been reported previously that entry into stationary phase generates a CpxA-independent response (11, 14). The nature of the response, however, has re-

mained elusive. The response of cells grown in glucose minimal media buffered with phosphate depends in part on σ^S (28), while the response of cells grown in LB does not (30). Like LB, TB is based on tryptone. Furthermore, σ^S is not fully functional this early in the transition to stationary phase, at least during growth in LB (142). Finally, deletion of both CpxA and the Pta-AckA pathway reduced growth-dependent *cpxP* activity to levels that resembled those of *cpxR* mutants (Fig. 5A). Thus, as cells enter stationary phase, *cpxP* becomes activated in response to both CpxA-dependent and Pta-AckA-dependent stimuli. This raises an intriguing question. During normal growth in a tryptone-based broth, why and how would CpxRA signaling respond to both envelope-associated and growth-associated cues?

Cells experience multiple stimuli that impact the phosphorylation status of RRs, including CpxR. Thus, the CpxR-dependent behavior of WT cells represents an integration of all these stimuli, and efforts to dissect this and other complex pathways must take this into account.

It is difficult to imagine that this complex signal integration is a privilege of the CpxAR pathway. In fact, evidence from the Wolfe laboratory suggests that at least one other 2CST pathway, the Rcs (Regulator of Capsule Synthesis) phosphorelay, also possesses a highly intricate and sophisticated mechanism to integrate signals that originate from the outer and inner membranes, periplasm and cytoplasm. The Rcs phosphorelay activates genes required for capsular biosynthesis (47) and multiple stresses (10, 26), while repressing genes required for flagellar biogenesis (37). Published evidence demonstrated that the RR RcsB can be both phosphory-

lated and acetylated, and that both modifications can affect the function of this protein (38, 130). RcsB phosphorylation can occur via the canonical SK-dependent phosphorylation or via an AcP-dependent mechanism (38, 146). Parallel to what I demonstrated for CpxR, multiple observations support the hypothesis that AcP-dependent RcsB phosphorylation occurs on the conserved Asp residue that functions as the phosphoacceptor for sensor kinase-dependent phosphotransfer (Hu, L. I. and Wolfe, A. J. unpublished data). It is the acetylation of RcsB that seems to challenge all previously established rules and concepts regarding protein acetylation. *In vitro,* acetylation can occur via an YfiQ-dependent transfer of the acetyl group from AcCoA to Lys-180 of the DNA-binding motif of RcsB (130). *In vitro,* however, the transfer of acetyl groups from AcCoA to RcsB also seems possible in the absence of any acetyltransferase (Hu, L. I. and Wolfe, A. J. unpublished data); this mechanism might be similar to the autoacetylation processes proposed for ACS and CheY (4). An additional autoacetylation mechanism seems to use AcP as the acetyl donor, Linda Hu possesses evidence that LpdA can be acetylated using acetyl groups from AcP and I demonstrated that the same can occur to RNAP (Chapter 5). In fact, recent data from the Wolfe laboratory demonstrate that AcP has a large impact on the overall protein acetylation status of *E. coli* (Bozena Zemaitaitis and Alan J. Wolfe, unpublished data). It remains to be determined how much of this impact is direct, as seems to be the case for RNAP and LpdA acetylation, and how much of the impact is indirect.

THE LOSS OF THE Pta-AckA PATHWAY

Disruption of the Pta-AckA pathway is likely to have many effects in *E. coli* metabolism and therefore the overall physiology of the cell. The Pta–AckA pathway does more than simply synthesize AcP. During acetogenesis, this pathway recycles CoA, generates ATP, and evolves acetate [reviewed in (144)]. *E. coli* produces limiting amounts of CoA; therefore, cells must recycle CoA to maintain glycolytic flux, and loss of the Pta-AckA pathway removes their "favored" option, at least at neutral pH, potentially leading to accumulation of AcCoA [review in (144)].

In many respects, mutants that lack AckA or both Pta and AckA behave similarly. In batch culture, they excrete little or no acetate, and they limit their production of ethanol. Instead, they both excrete pyruvate, lactate, and glutamate, a behavior that might be explained by the observation that these mutants exhibit increased expression and activity of key glycolytic and TCA cycle enzymes [reviewed in (144)]. Surprisingly, they do not increase expression of their CoA biosynthetic machinery (148), nor do they alter their AcCoA pool relative to that of their WT parent (72). The increased expression and activity of both glycolytic and TCA cycle enzymes and the altered excretion profile suggest that cells attempt to compensate for the loss of the Pta-AckA pathway (148). This loss of the pathway by the cells and their attempt to compensate for it can make the interpretation of some phenotypes associated with AcP a little challenging. In fact, some studies provide some evidence for pathway involvement but fail to make a strong connection to AcP [for a review, see reference (145)]. Similarly to what I found for CpxAR signaling, some further in-

vestigation of these pathways may discover new mechanisms that involve more than just AcP-dependent phosphorylation of RRs and might also involve more than just 2CST.

AcP-DEPENDENT PHOSPHORYLATION

Plenty of evidence exists that AcP can readily phosphorylate RRs *in vitro* (144). *In vivo,* AcP-dependent behaviors have been reported in diverse bacteria, including pathogens [for example, (99, 150)]. In *E. coli* for example, the regulation of about approximately 100 genes correlates with the status of AcP (148). However, the evidence for a transfer of phosphoryl group from AcP to some RR, in many cases, is either weak or nonexistent [reviewed in (146)]. Some of the challenges in demonstrating the role of AcP as a global signaling molecule comes from the pivotal role AcP plays in central metabolism, which has made the interpretation of some of the phenotypes related to disruption of the Pta-AckA pathway challenging. Additional challenges can be attributed to the labile nature of the acyl phosphate bond present in phospho-Asp modification (1), which has hindered the development of antibodies capable of detecting phosphorylated RRs and made it difficult to obtain definitive evidence of an AcP-dependent phosphorylation of RR *in vivo.*

Relatively recent biotechnological advances have the potential to facilitate the detection of phosphorylated RRs *in vivo* and allow for a more definitive test of the hypothesis that AcP acts as a phosphoryl donor to RRs *in vivo*. In 2006, Kinoshita *et al.* optimized a method to detect phosphorylated proteins by mobility shift assay.

This method consists of introducing a phosphate-binding tag (Phos-Tag™) bound to acrylamide beads into SDS-PAGE gels. The Phos-Tag™ molecules, with the help of Zn^{2+} or Mn^{2+}, preferentially interact with molecules with phosphomonoester bonds as seen in proteins with phosphorylated Ser, Thr and Tyr side chain residues, slowing the electrophoretic migration of these proteins through a gel (70). In 2008, Barbieri and Stock utilized phos-tag™ to detect the labile phosphorylation of the *E. coli* RR PhoB both *in vitro*, using purified protein, and *in vivo,* using cell lysates (5). Using this methodology, Liu *et al.* demonstrated that, consistent with the notion that CpxA function as a phospho-Asp phosphatase *in vivo*, phosphorylation of *Yersinia pseudotuberculosis* CpxR, expressed from a plasmid vector, increased in a *cpxA* mutant background when compared to the WT parent. This finding corroborates what has been demonstrated *in vitro* (36, 111) and proposed *in vivo* (6, 17, 23, 24, 149). Liu *et al.* (2011) also demonstrated that this CpxA-independent phosphorylation required the Pta-AckA pathway, but failed, however, to perform a complete epistasis analysis (85).

I extended the results of Liu *et al* (2011) by demonstrating that, in *E. coli*, endogenously expressed CpxR becomes phosphorylated in a glucose-dependent manner independently of the SK CpxA. Using a plasmid-born CpxR, I demonstrated that the glucose-induced phosphorylation requires the conserved Asp-51 residue. I also conducted a complete epistasis analysis of the Pta-AckA pathway effect on glucose-induce CpxR phosphorylation and concluded that this phosphorylation requires AcP. This conclusion was based on the presence of phosphorylated CpxR

when cells accumulated AcP, whereas complete deletion of the Pta-AckA pathway led to almost undetectable levels of phospho-CpxR. However, I acknowledge the existence of a weak glucose- and AcP-independent signal exhibited by the *pta ackA* double mutant. Currently, we do not know the origin of this weak signal. Perhaps, under the conditions tested, a small percentage of CpxR is phosphorylated by a non-cognate sensor kinase and/or another small phospho-donor.

Lastly, while the use of Phos-Tag™ requires some optimization, at the present moment, this is likely the best and most accessible approach to detect phosphorylated RRs *in vivo*.

PROTEIN ACETYLATION IS AN IPORTANT CONTRIBUTOR TO *cpxP* TRANSCRIPTION

In addition to being regulated via CpxA- and/or AcP-dependent phosphorylation, I propose that *cpxP* activity also is regulated via an N^{ε}-lysine acetylation.

Exposure to glucose can induce *cpxP* transcription in a sensor kinase-independent manner. While one might initially consider the involvement of catabolite repression in glucose-induced *cpxP* transcription, the observation that both catabolite-repressing and non-catabolite-repressing carbon sources (e.g. glucose and acetate, respectively) can trigger CpxA-independent *cpxP* transcription argues against it. Instead, this finding focused my attention on the alternative hypothesis that transcription-activating carbon sources contribute to *cpxP* transcription via an acetylation event. I base this proposition on the following evidence: (1) growth in

the presence of excess glucose leads to an increase in the overall protein acetylation status that can be detected via mass spectrometry analysis and Western immunoblot analysis. (2) exposure to the NAD^+-dependent deacetylase inhibitor nicotinamide induces *cpxP* transcription in the absence of glucose or other carbon sources, (3) overexpression of the NAD^+-dependent deacetylase CobB inhibits glucose-induced *cpxP* transcription, and (4) glucose-induced *cpxP* transcription specifically requires the GCN5-like acetyltransferase YfiQ and a lysine residue 298 located on the surface of the αCTD that, under my tested growth condition, is acetylated in a glucose- and YfiQ-dependent manner.

Although I demonstrated that glucose-induced *cpxP* transcription is affected by CobB and YfiQ, the mechanism behind this effect remains unclear. Exposure to glucose altered the acetylation status of multiple proteins. This was observed as an increase in the number and/or intensity of acetylated bands as detected by Western immunoblot analysis. Glucose also induced a two-fold increase in the number of proteins and lysines detected to be acetylated by mass spectrometry (B. Schilling, B. Gibson, and A. J. Wolfe, unpublished data). Unlike glucose, the CobB inhibitor nicotinamide caused relatively few changes in the total acetylation profile, as previously reported (157). Thus, nicotinamide and therefore CobB inhibition likely exert a more limited effect on the *E. coli* acetylome than does glucose. This marginal effect of CobB on overall protein acetylation was quantified by mass spectrometry analysis. In general, deletion of CobB promoted a 10% increase in overall protein acetylation in the absence of glucose and approximately 20% when the cells were grown in

the presence of glucose (B. Schilling, B. Gibson, and A. J. Wolfe, unpublished data). Several factors could contribute to this observation: (1) the deacetylase CobB might have a limited number of acetylated targets, whereas the additional AcCoA could be used as an acetyl donor by multiple acetyltransferases; (2) CobB might not be fully expressed and active when the samples were collected for analysis; and (3) presently unidentified deacetylases could exist (L. I. Hu and A. J. Wolfe, unpublished data). Under any of these circumstances, inhibition of the deacetylase CobB would not be expected to exert a large impact on the acetylome. It should be noted, however, that CobB overexpression antagonized glucose-induced acetylation. This behavior is consistent with one of three possibilities: (1) CobB could directly reverse glucose-induced acetylations, (2) CobB could activate another deacetylase(s) that reverses those acetylations, or (3) CobB could inhibit the acetyltransferase(s) responsible for those acetylations.

Glucose-induced *cpxP* transcription requires YfiQ, and YfiQ is required for approximately 75% of the glucose-induced Lys acetylation on RNAP; however, I was unable to obtain evidence that YfiQ can directly acetylate RNAP *in vitro*. This inability to directly acetylate RNAP with YfiQ *in vitro* makes me favor the possibility that the YfiQ effect on RNAP acetylation is indirect. Indeed, the Wolfe laboratory possesses evidence that YfiQ inhibits glycolysis, which would diminish the concentration of AcP (A. J. Walker-Peddakotla, M. Kuhn, W. F. Anderson and A. J. Wolfe, unpublished data).

The pattern of RNAP acetylation is affected by disruption of the Pta-AckA pathway. One of the key residues under these conditions is α Lys-291. Under conditions that promote Lys-291 acetylation, *cpxP* transcription is diminished. Contrary to what I observed for Lys-298, I found no evidence that YfiQ plays any role in Lys-291-dependent inhibition of *cpxP* transcription. Although overexpression of YfiQ promoted a small increase in glucose-induced *cpxP* transcription, it did not alter the weakened response by the *ackA* mutant. This weakened response also was unaffected by deletion of *yfiQ*. Although, overexpression of several acetyltransferases inhibited *cpxP* transcription in the WT parent, deletion of any single enzyme did not permit the *ackA* mutant to respond robustly to glucose. A simple explanation for these results is that multiple acetyltransferases could acetylate Lys-291; however, at this point, I have identified no acetyltransferase that could acetylate RNAP. Alternatively, Lys-291 could become acetylated independently of the action of an acetyltransferase. Efforts to distinguish between these two mechanisms are underway. Indeed, quantitative mass spectrometry revealed that Lys-291 is hyperacetylated in the glucose-exposed *ackA* mutant relative to its WT parent (B. Schilling, B. Gibson, and A. J. Wolfe, unpublished data).

To date, YfiQ is the only *E. coli* acetyltransferase with confirmed protein acetyltransferase activity. However, under the conditions I tested (TB supplemented with 0.4% glucose), YfiQ only affected the acetylation of approximate 10% of proteins when compared to WT, as detected by mass spectrometry analysis (B. Schil-

ling, B. Gibson, and A. J. Wolfe, unpublished data). Some of the proteins acetylated by YfiQ are proteins involved in the switch between glycolysis and gluconeogenesis, leading to the hypothesis that YfiQ contributes to the regulation of central metabolic pathways. Indeed, in support to this hypothesis, YfiQ mutants possess a growth defect under certain limiting nutritional conditions, when compared to WT (Walker-Peddakotla, A. J. and Wolfe A. J., unpublished). Changes in carbon sources that are supplemented into the media can alter the acetylation patterns of more than 100 proteins in both *E. coli* and *Salmonella.* This change in acetylation has been proposed to lead to change in the direction of carbon flux to glycolysis or gluconeogenesis and control the activity of the glyoxylate shunt relative to the tricarboxylic acid cycle [reviewed in (132)]. Thus, it is possible that the YfiQ effect on RANP acetylation could be derived from an YfiQ effect in central metabolism.

The link between central metabolism and YfiQ goes beyond the YfiQ involvement in the acetylation of some metabolic proteins. Recent evidences suggest that, in *E. coli*, YfiQ transcription is affected by the concentration of cyclic AMP, linking transcription regulation of this acetyltransferase to central metabolism. It was proposed that this regulation occurs via CRP, as the putative *yfiQ* promoter contains two CRP binding elements that are required for *yfiQ* transcription (18). In contrast, in *M. tuberculosis*, cAMP directly activates the YfiQ homologue Mt-PatA, by binding to a cyclic nucleotide–binding domain fused to the N terminus of the catalytic PAT domain (75, 96). Efforts to understand the connection between YfiQ, cAMP, central metabolism, and protein acetylation are underway.

AcP-DEPENDENT ACETYLATION

The data presented here support the hypothesis that AcP affects the overall protein acetylation status of *E. coli*. RNAP isolated from *ackA* mutant cells exhibited a vastly different acetylation pattern than RNAP isolated from the WT parent**.** A similar observation has been made for RcsB (L. I. Hu and A. J. Wolfe, unpublished data). On the basis of these observations, anti-acetyl-lysine Western blot and mass spectrometric analyses were performed on an isogenic set of strains that included mutants defective for the Pta-AckA pathway and the known acetylation-associated enzymes (B. Zemaitaitis, B. Schilling, B. Gibson, and A. J. Wolfe). Western blot analysis provided evidence that AcP is a major regulator of protein acetylation in *E. coli.* Whenever AcP levels were increased (i.e., in an *ackA* mutant grown in TB or in a *pta* mutant gorwn in TB supplemented with acetate, or in WT cells grown in TB supplemented with glucose), global acetylation increased. Mass spectrometric analysis extended and quantified those observations. For example, the number of proteins detected as acetylated by mass spectrometry analysis was 90% greater in cell lysates from the *ackA* mutant than from its WT parent. This increase in protein acetylation was observed whether glucose was present or not. Consistent with the notion that this increase in protein acetylation is an AcP-dependent phenotype, the *pta ackA* double mutant exhibited a major decrease in the total number of detected acetylated proteins to a total of only 60% of the WT level, when the cells were

grown in the absence of glucose. Finally, Western immunoblot analyses showed that most of the glucose-dependent acetylation required AcP.

This AcP-dependent increase in detectable acetylated proteins could be explained by a combination of any of the following possibilities: (1) AcP could induce an increase in the expression and/or activity of an AcCoA-dependent protein acetyltransferase, (2) AcP could function as an acetyl donor for a presently unidentified acetyltransferase, (3) or AcP could directly transfer its acetyl group to the target protein.

Whichever the mechanism, initial analyses demonstrate that approximately 7% of total *E. coli* proteins are acetylated in the *ackA* mutant. This number jumps to approximately 12% when the cells are grown in the presence of glucose. This number is likely to be an underestimate as this represents a snapshot of the acetylome as the cells enter stationary phase. Additionally, due to inherent limitations of the approach used, proteins found in very low concentration may not have been detected by this mass spectrometry analysis, which would also contribute to an underestimate of the total number of acetylated proteins.

RNAP is among some of the proteins whose acetylation has been shown to be affected by disruption of the Pta-AckA pathway. This evidence comes from mass spectrometric analysis of RNAP purified from immunoprecipitation and also from RNAP that present in cell lysates. Although my results implicated approximately 75% of the acetylation sites on RNAP to YfiQ, my attempts to reconstitute this reaction *in vitro* with purified proteins have failed. Instead, as detected by Western im-

munoblot analysis, *in vitro* acetylation of some RNAP subunits occurred when AcP was added to the reaction as the acetyl donor. Since I cannot completely discard the possibility that my purified protein samples could be contaminated with a protein that uses AcP as an acetyl donor to acetylate proteins, my finding that addition of AcP to purified RNAP leads to its acetylation lends support to two of the three possibilities presented above: (a) An acetyltransferase could use AcP to acetylate proteins *in vivo*, or (b) AcP could acetylate these proteins directly. Lastly, it is important to mention that although I was unable to acetylate RNAP *in vitro* using AcCoA as the acetyl donor and YfiQ as the acetyltransferase, I cannot discard the possibility that this reaction may occur *in vivo* and that I may have not found the appropriate *in vitro* condition to reproduce it.

It remains untested if the AcP-dependent acetylation of RNAP has any effect on its activity *in vivo*. While the data I presented here support the hypothesis that acetylation of Lys-298 and Lys-291 contributes to the regulation of *cpxP* transcription, I cannot yet say whether the acetyl group for these acetylation events come from AcCoA or from AcP. Mass spectrometry analysis of *in vitro* acetylated RNAP is currently underway to determine if AcP can acetylate either of these residues *in vitro*. Independently of where the acetyl groups for acetylation of Lys-298 and Lys-291 come from, I did observe an AcP-dependent effect on RNAP activity *in vitro*, marked by a progressive decrease in *rna1* transcription as the concentration of AcP in the reaction increased. This effect is likely to be context dependent as AcP had the

opposite effect on *cpxP* transcription, leading to a dose-dependent increase in *cpxP* transcription.

If AcP can indeed directly acetylate proteins *in vivo,* one could easily imagine that if not properly regulated this acetylation could have some negative impact on overall cellular physiology. One likely candidate to help the cells regulate AcP-dependent acetylation is the deacetylase CobB. In an *in vitro* assay, purified CobB has been shown to be capable of deacetylating short peptides that had been previously acetylated using AcP as the acetyl donor (M. Kuhn, M. Scholle, M. Mrksich, W. F. Anderson, A. J. Wolfe, unpublished data). Thus, it is possible that, in a manner parallel to the phospho-Asp phosphatase activity of some SKs, the deacetylase CobB may help maintain the proper levels of certain acetylated proteins by deacetylating proteins that have been previously acetylated by AcP. Indeed, evidence from the Wolfe laboratory supports the hypothesis that CobB deacetylates some AcP-dependent lysine acetylations (A. AbouElfetouh, D. Christensen, and A. J. Wolfe, unpublished data).

A ROLE FOR ACETYLATED Lys-298

Following enrichment by immunoprecipitation, mass spectrometry analysis revealed acetylation of several surface-exposed lysines of the β, β', and α subunits of RNAP isolated from WT cells exposed to glucose. Due to its smaller size and the fact that only two lysines were found to be acetylated, I further investigated the effect of αCTD acetylation on *cpxP* transcription. Using a merodiploid system, I asked wheth-

er any of the Lys on αCTD played a substantial role in glucose-induced *cpxP* transcription and found that Lys-298 did. Expression of the K298A mutant inhibited transcription, whereas similar expression of the other α K-to-A mutants, including the alanine mutant of Lys-297, which was also found to be acetylated, had no significant effect on glucose-induced *cpxP* transcription. Furthermore, the requirement for Lys-298 was specific: extracytoplasmic stimuli that activate CpxA kinase activity and thus rapid phosphorylation of CpxR did not require Lys-298.

The findings presented here substantially add to data presented by previous reports that RNAP subunits can become acetylated (155, 157). It also establishes at least one growth condition under which these acetylation events likely occur and implicate the acetyltransferase YfiQ in acetylation of the majority of those sites, including Lys-1200 of β, which had been previously reported to be acetylated (157). However, at this moment it is not clear if YfiQ is a direct or indirect contributor to these acetylations.

It is also unclear what percentage of α becomes acetylated on Lys-298 under the conditions tested, since this value has to be obtained by quantitative mass spectrometry methods. However, clues exist to suggest that the majority of α isolated from glucose-exposed WT cells is acetylated. These clues come from the fact that trypsin, the protease used to obtain the MS-analyzed peptides, cleaves after lysines but not after acetyllysines. From glucose-naïve WT cells, we detected the non-acetylated peptides (291-297) TPNLGK and (299-310) SLTEIKDVLASR, suggesting that Lys-297 and Lys-298 were not acetylated. In contrast, from glucose-exposed

WT cells, our collaborators detected the related but acetylated peptides (291-298) TPNLGK297(+42)K and (298-304) K298(+42)SLTEIK, suggesting that Lys-297 and Lys-298 could be acetylated. Strikingly, the inability to detect the non-acetylated peptides TPNLGK and SLTEIKDVLASR in glucose-exposed samples suggests that much of the α isolated from glucose-exposed WT cells is acetylated on Lys-297 and Lys-298.

How could acetylation of Lys-298 affect *cpxP* transcription? Analysis of the crystal structure of CRP and αCTD bound to a synthetic DNA fragment containing two CRP binding sites flanked by αCTD binding sites suggests that Lys-298 of α makes direct interaction with DNA adjacent to the CRP binding site (7). Thus, Lys-298 likely plays a role in closed complex formation, the first step in transcription initiation. So how could acetylation of Lys-298 work? While I cannot definitively answer how acetylation of Lys-298 contributes to *cpxP* transcription, it is possible that non-acetylated αCTD binds to a site that is not conducive to CpxR-dependent activation. If so, the acetylation of Lys-298 could re-position the αCTD, permitting it to make productive contact with CpxR. Alternatively, CpxR and RNAP could form an overly stable complex that binds tightly to the *cpxP* promoter. If so, then acetylation of Lys-298 might loosen this interaction, facilitating promoter clearance. Whichever mechanism is true, it should be noted that although Lys-298 is required for glucose-induced *cpxP* transcription, it may not be sufficient; other acetylated lysines on the surface of the other RNAP subunits might also participate. Additionally, Lys-298 is

not required for CpxA-dependent activation of *cpxP*, suggesting a distinct activation mechanism.

Other questions remain: What are the general rules for activation? How general is the effect of Lys-298 acetylation? Does protein acetylation influence transcription from other CpxR-dependent promoters? Does it influence CpxR-independent promoters? There is evidence that the response to glucose and other carbon sources is context-dependent, i.e. some CpxR-dependent promoters respond to carbon source exposure and some do not (A. Cosgrove, B. P. Lima and A. J. Wolfe, unpublished data). This context-dependence suggests that the mechanism(s) by which acetylation influences transcription is not simple, and may involve factors such as promoter architecture and nucleoprotein complex composition.

A ROLE FOR ACETYLATED Lys-291

The acetylation profile of α isolated from the *cpxA ackA* mutant differed from the acetylation profile of α isolated from its WT parent. Thus, it appears that RNAP can become acetylated on multiple lysine residues on different subunits and that the acetylation pattern of RNAP can differ depending on genetic background and growth condition. While one α acetylation was common to both strains, five acetylations were specific to the *cpxA ackA* mutant including Lys-291, which had been shown by other to be acetylated (ref), and only one was specific to WT (i.e., Lys-298). The genetic screen conducted here suggests that Lys-291 is definitely involved in weakening the response to 0.4% glucose by the *ackA* mutant or to 4.0% glucose by its WT

parent. I currently do not possess definitive evidence that acetylation of Lys-291 causes these behaviors; however, support for such a mechanism is provided by the dramatic reduction in glucose-induced *cpxP* transcription by the K291Q mutation, which is predicted to exhibit properties that mimic acetylated Lys-291 (25). While the underlying molecular mechanism remains to be determined, it is distinctly possible that Lys-291 acetylation could affect one of the many interactions made by α and, as a result, decrease the stability of the transcription complex. Lys-291 is located on the αCTD helix-hairpin-helix motif, which is reported to contribute to both nucleic acid binding and protein-protein interaction (117). To the best of my knowledge, no evidence exists that Lys-291 makes direct contact with DNA. In contrast, Lys-291 has been reported to make direct contact with activation region 2 of the transcription antiterminator NusA (116). Furthermore, Lys-291 is located near the 287 determinant, an αCTD surface known to make direct contact with the catabolite activator protein (CAP, also known as CRP) [reviewed in (14)]. Although no direct evidence exists for an αCTD-CpxR interaction, our report that the αCTD is required for glucose-induced *cpxP* transcription (83) hints that an interaction between these two proteins might exist, perhaps in manner similar to that reported for OmpR, another member of the winged-helix-turn-helix family of response regulators [reviewed in (67)].

Using the well-characterized CRP-αCTD interaction as a guide, we hypothesize that Lys-291 might participate in an interaction between CpxR and the αCTD. If so, then the acetylation of Lys-291 could affect this interaction and therefore impact

transcription. Efforts are underway to identify the mechanism by which Lys-291 and its acetylation affect *cpxP* transcription.

MODEL

I envision a simple model that accounts for all the observations presented here. When a cell grows in buffered TB, the lack of a strong extracytoplasmic stimulus permits CpxA to function as a net phosphatase, removing phosphoryl groups from phospho-CpxR. If that cell becomes exposed to a carbon source (e.g. glucose, pyruvate, lactate or acetate) whose metabolism results in an increase in intracellular AcP concentration, then the increased AcP concentration leads to phosphorylation of certain RRs (including CpxR) and to acetylation of more than 10% of total *E. coli* proteins, including proteins that comprise RNAP. If metabolism of that carbon source (e.g. glucose or lactate) also requires an NAD^+-dependent enzyme (e.g. glyceraldehyde dehydrogenase or lactate dehydrogenase, respectively), then a competition might ensue with the NAD^+-dependent deacetylase CobB for the limited supply of NAD^+. This competition would result in reduced deacetylation of CobB targets. The result would be a dramatic increase in the acetylation of certain proteins, including many that regulate transcription.

YfiQ influences the pattern of RNAP acetylation; however, the mechanism remains unknown. One possibility could be that a YfiQ-dependent effect on carbon flux through central metabolism could influence AcP concentration, and thus lead to changes in the acetylation pattern of RNAP. Distinct acetylation sites on RNAP could

differentially impact its activities, including its ability to bind to promoters and to interact with transcription factors. The outcome of RNAP acetylation at the level of *cpxP* transcription seems to be either activation (if Lys-298 is acetylated) or inhibition (if Lys-291 is acetylated).

Several important questions remain unanswered. Does YfiQ directly acetylate RNAP and does this acetylation affect *cpxP* transcription. How does AcP affect the overall pattern of protein acetylation? Do acetyltransferases exist that can utilize AcP as the acetyl donor? Does the *in vitro* acetylation of RNAP by AcP represent a situation that occurs *in vivo*? CobB impact on overall protein acetylation seems to have been modest. Is this a clue that other unidentified protein deacetylases exit in *E. coli*? What is the overall transcriptional impact of RNAP acetylation?

In summary, I propose that AcP can function as a phosphoryl donor to CpxR, both *in vitro* and *in vivo* and an acetyl donor to RNAP at least *in vitro* and probably *in vivo*. The output of this multiple modification, at the level of the *cpxP* promoter, seems to be modulation of the strength of *cpxP* transcription. My work suggests that acetylation of Lys-298 somehow contributes to promoter activity, whereas acetylation of Lys-291 dampens this activation.

CONCLUDING REMARKS

Great strides have been made to understand the role of protein acetylation in eukaryotes. In contrast, our knowledge of protein acetylation in bacteria remains in its infancy. Our current knowledge is restricted to three lists of acetylated bacterial

proteins, the structures of several bacterial GNATs, some functions of a single acetyltransferase (YfiQ/Pat), a single deacetylase (the NAD^+-dependent sirtuin CobB), and the effects of acetylation on the functions of three acetylated proteins that function in central metabolism, chemotaxis [reviewed in (53)] and RNA metabolism (80, 81). The data presented here extends that knowledge by (1) implicating AcP as a major determinant in controlling protein acetylation, (2) showing that several RNAP subunits can become acetylated on multiple lysines, and (3) demonstrating that the protein acetylation machinery of *E. coli* affects transcription of at least one promoter, potentially by its ability to modify RNAP, the molecular machine that performs transcription of all bacterial genes.

Table 1

Strain and plasmid Table

Strain	Relevant Characteristic	Source/Reference
PAD282	F- *araD139* Δ(*argF-lac*)*U169* *rpsL150*(Str^r) *relA1 flhD5301 deoC1* λΦ(*PcpxP'-lacZ*)	(30)
PAD292	PAD282 *cpxR1*::*spc* (spectinomycin insertion in *cpxR* with polar effect on *cpxA*)	(30)
PAD348	PAD282 *cpxA*::cam	DiGuiseppe & Silhavy (Princeton University)
AJW2790	PAD282 *ackA::kan*	(149)
AJW2791	PAD282 Δ(*ackA pta*)::*cam*	(149)
AJW2794	PAD282 *cpxA*::cam *ackA::kan*	(149)
AJW3143	PAD282 *yfiQ::kan*	(83)
AJW3827	PAD292 *ackA::kan*	(84)
AJW3875	PAD292 Δ(*ackA pta hisJ hisP dhu*) *zej223::Tn*10	(84)
AJW3167	PAD282 Δ*cpxA::kan*	(84)
AJW3994	PAD282 Δ*ackA::frt*	(83)
AJW4303	AJW3994 Δ*tmcA::kan*	(84)
AJW4304	AJW3994 Δ*ypeA::kan*	(84)
AJW4305	AJW3994 Δ*yedL::kan*	(84)
AJW4306	AJW3994 Δ*aat::kan*	(84)
AJW4307	AJW3994 Δ*yhbS::kan*	(84)
AJW4308	AJW3994 Δ*yafP::kan*	(84)
AJW4309	AJW3994 Δ*yiiD::kan*	(84)
AJW4310	AJW3994 Δ*rimJ::kan*	(84)
AJW4311	AJW3994 Δ*rimL::kan*	(84)
AJW4312	AJW3994 Δ*yiaC::kan*	(84)
AJW4313	AJW3994 Δ*yjhQ::kan*	(84)
AJW4314	AJW3994 Δ*speG::kan*	(84)
AJW4315	AJW3994 Δ*argA::kan*	(84)

AJW4316	AJW3994 Δ*rimI::kan*	(84)
AJW4317	AJW3994 Δ*yhhY::kan*	(84)
AJW4318	AJW3994 Δ*phnO::kan*	(84)
AJW4319	AJW3994 Δ*yjdJ::kan*	(84)
AJW4320	AJW3994 Δ*yjaB::kan*	(84)
AJW4321	AJW3994 Δ*wecD::kan*	(84)
AJW4322	AJW3994 Δ*yjgM::kan*	(84)
AJW4333	AJW3994 Δ*yncA::kan*	(84)
AJW4867	PAD282 Δ*cpxA::kan* Δ*ackA::frt*	P1:JW3882 → AJW3994 (KmR)
AJW1939	*ackA::kan*	(73)
AJW2013	Δ(*ackA pta hisJ hisP dhu*) zej223::Tn10	(148)
JW2293	Δ*ackA*::*kan*	(2)
JW3882	Δ*cpxA::kan*	(2)

Plasmid	**Relevant Characteristics**	**Source/Reference**
pCA24n	Control plasmid (Cm^R)	(71)
pCA24n-*cpxR*	Plasmid expressing 6xHis-CpxR under the control of an IPTG-inducible promoter (Cm^R)	(71)
pCA24n-*cpxRD51A*	Plasmid expressing 6xHis-CpxRD51A under the control of an IPTG-inducible promoter (Cm^R)	(84)
pBPL001	pET-28 derivative expressing 6xHis-CpxR under the control of an IPTG-inducible promoter. (Kn^R)	(84)
p770-*cpxP*	p770 derivative expressing *cpxP* promoter (Ap^R)	Unpublished
pCA24n-*yfiQ*	Plasmids expressing 6xHis-*yfiQ* under the control of an IPTG-inducible promoter (Cm^R)	(71)
pCA24n-*yiiD*	Plasmids expressing 6xHis-*yiiD* under the control of an IPTG-inducible promoter (Cm^R)	(71)
pCA24n-*atoB*	Plasmids expressing 6xHis-*atoB* under the control of an IPTG-inducible promoter (Cm^R)	(71)

pCA24n-*argA*	Plasmids expressing 6xHis-*argA* under the control of an IPTG-inducible promoter (Cm^R)	(71)
pCA24n-*elaA*	Plasmids expressing 6xHis-*elaA* under the control of an IPTG-inducible promoter (Cm^R)	(71)
pCA24n-*yjgM*	Plasmids expressing 6xHis-*yjgM* under the control of an IPTG-inducible promoter (Cm^R)	(71)
pCA24n-*yjaB*	Plasmids expressing 6xHis-*yjaB* under the control of an IPTG-inducible promoter (Cm^R)	(71)
pCA24n-*yhhY*	Plasmids expressing 6xHis-*yhhY* under the control of an IPTG-inducible promoter (Cm^R)	(71)
pCA24n-*rimJ*	Plasmids expressing 6xHis-*rimJ* under the control of an IPTG-inducible promoter (Cm^R)	(71)
pCA24n-*yjdJ*	Plasmids expressing 6xHis-*yiid* under the control of an IPTG-inducible promoter (Cm^R)	(71)
pPHB1	pGEM-T-Easy derivative expressing *phbCAB*$^+$ (Ap^r)	(83)
pPHB2	pTAC-85 derivative expressing *phbCAB*$^+$ (Ap^r)	(83)
pREII	Plasmid expressing WT *rpoA* or alanine substitution derivatives (Ap^R)	(39)
pREII-*rpoAK10A*	pREII derivative expressing *rpoAK10A*	(84)
pREII-*rpoAK25A*	pREII derivative expressing *rpoAK25A*	(84)
pREII-*rpoAK95A*	pREII derivative expressing *rpoAK95A*	(84)
pREII-*rpoAK271A*	pREII derivative expressing *rpoAK271A*	(39)
pREII-*rpoAK291A*	pREII derivative expressing *rpoAK291A*	(39)
pREII-	pREII derivative expressing *rpoAK297A*	(39)

rpoAK297A		
pREII-*rpoAK298A*	pREII derivative expressing *rpoAK298A*	(39)
pREII-*rpoAK304A*	pREII derivative expressing *rpoAK304A*	(39)
pREII-*rpoAK291R*	pREII derivative expressing *rpoAK291R*	(84)
pREII-*rpoAK291Q*	pREII derivative expressing *rpoAK291Q*	(84)

Table 2.
The number of acetylated β and β' peptides detected from *ackA* mutant cells [1]

Beta (Glucose-exposed)	Replicate 1	Replicate 2	Replicate 3
Total Spectra	366	382	305
(R)EAPEGTVk$_{115}$(+42)DIK(E)	1	0	0
(R)QLEk$_{279}$(+42)DDVK(L)	1	1	0
(K)LSQSGHk$_{331}$(+42)R(I)	0	0	1
(R)Sk$_{639}$(+42)GESSLFSR(D)	0	1	1
(R)AVAVDSGVTAVAk$_{719}$(+42)R(G)	1	0	0
(R)DTk$_{844}$(+42)LGPEEITADIPNVGEAALSK(L)	1	0	0
(K)VTPk$_{890}$(+42)GETQLTPEEK(L)	1	1	1
(R)AIFGEk$_{909}$(+42)ASDVK(D)	1	1	1
(K)ASDVk$_{914}$(+42)DSSLR(V)	1	1	1
(R)ALEIEEMQLk$_{954}$(+42)QAK(K)	1	1	0
(R)AVLVAGGVEAEk$_{988}$(+42)LDK(L)	2	1	1
(K)LDk$_{991}$(+42)LPR(D)	1	0	0
(R)k$_{1035}$(+42)ITQGDDLAPGVLK(I)	0	1	1
(R)IQPGDk$_{1065}$(+42)MAGR(H)	1	0	0
(R)HGNk$_{1073}$GVISK(I)	0	1	1
(K)INAmLk$_{1133}$(+42)QQQEVAK(L)	1	2	2
(K)QQQEVAKk$_{140}$(+42)LR(E)	1	1	1
(R)k$_{1178}$(+42)GMPIATPVFDGAK(E)	1	2	2
(K)ELLk$_{1200}$LGDLPTSGQIR(L)	0	1	0

Beta' (Glucose-exposed)	Replicate 1	Replicate 2
Total Spectra	314	248
(K)AQTk$_{13}$(+42)TEEFDAIK(I)	1	1
(R)GLATTIk$_{395}$(+42)AAK(K)	1	1
(R)ITEYEk$_{557}$(+42)DANGELVAK(T)	1	0
(K)TSLk$_{570}$(+42)DTTVGR(A)	1	1
(R)AAAESSIQVk$_{953}$(+42)NK(G)	0	1
(K)GSIk$_{959}$(+42)LSNVK(S)	1	1

[1]**Red, acetylated peptides detected in at least two biological replicates; Black, acetylated peptides detected in one biological replicate; m, oxidized methionine, M, methionine; k, acetylated lysine; K, lysine.**

Table 3.
ACETYLATED α PEPTIDES FROM *ackA* MUTANT CELLS[1].

Peptide[2]	Start	Stop	Acetylated Lysine	Glucose-exposed Replicate 1 S3C[3]	Glucose-exposed Replicate 2 S3D	Glucose-Naïve Replicate 1 S3E	Glucose-Naïve Replicate 2 S3F	Glucose-Naïve Replicate 3 S3G
(-)mQGSVTEFLK(P)	1	10		1	3	1	0	1
(-)MQGSVTEFLKPR(L)	1	12		8	28	4	3	6
(-)mQGSVTEFLkPR(L)	1	12	10	0	1	0	0	1
(R)LVDIEQVSSTHAK(V)	13	25		10	20	10	12	13
(R)LVDIEQVSSTHAKVTLEPLER(G)	13	33		0	0	0	2	1
(R)LVDIEQVSSTHAkVTLEPLER(G)	13	33	25	0	1	0	0	0
(R)VQGKDEVILTLNK(S)	92	104		139	137	153	90	24
(R)VQGkDEVILTLNK(S)	92	104	95	2	1	0	0	0
(R)TEVELLK(T)	285	291		4	9	7	9	7
(R)TEVELLKTPNLGK(K)	285	297		0	1	0	2	4
(R)TEVELLkTPNLGK(K)	285	297	291	2	1	0	0	0
(K)TPNLGkK(S)	292	298	297	2	3	0	0	0
(K)KSLTEIK(D)	298	304		0	3	1	2	0
(K)KSLTEIKDVLASR(G)	298	310		0	0	0	1	3
(K)kSLTEIKDVLASR(G)	298	310	298	0	0	0	1	0
(K)SLTEIKDVLASR(G)	299	310		7	9	8	9	9
(K)SLTEIkDVLASR(G)	299	310	304	1	1	0	0	0
			Total Spectra	315	486	298	216	176

[1] Green, unacetylated peptides; Red, acetylated peptides
[2] m, oxidized methionine; M, methionine; k, acetylated lysine; K, lysine
[3] Supplemental Table in which the raw data is presented

TABLE 4

Lysine-acetylated β peptides identified from glucose-exposed wild-type cells

Sequence	Prob	SEQUEST XCorr	SEQUEST ΔCn	Observed	Actual Mass	Charge	Delta PPM
(R)DNK$_{236}$(+42)LQmELVPER(L)	95%	2,955	0.481	510,5923	1.528,76	3	-0,4136
(R)QLEK$_{279}$(+42)DDVK(L)*	95%	2,407	0.4241	508,7665	1.015,52	2	-0,3027
(K)VTPK$_{890}$(+42)GETQLTPEEK(L)	95%	3,296	0.6551	799,9181	1.597,82	2	0,8714
(R)AIFGEK$_{909}$(+42)ASDVK(D)*	95%	3,424	0.6959	603,8215	1.205,63	2	-0,7829
(K)ASDVK$_{914}$(+42)DSSLR(V)*	95%	3,105	0.3712	560,2856	1.118,56	2	-0,08906
(R)ALEIEEmQLK$_{954}$(+42)QAK(K)	95%	3,883	0.6378	786,9191	1.571,82	2	0,2837
(R)AVLVAGGVEAEK$_{988}$(+42)LDK(L)	95%	4,315	0.7414	770,9325	1.539,85	2	-0,3886
(K)LDK$_{991}$(+42)LPR(D)*	95%	1,741	0.3937	392,2397	782,4648	2	-0,5344
(R)K$_{1035}$(+42)ITQGDDLAPGVLK(I)	95%	3,136	0.5483	748,9197	1.495,82	2	-0,07396
(R)mNIGQILETHLGmAAK$_{1122}$(G)	95%	3,105	0.4271	600,9708	1.799,89	3	-0,3436
(K)INAmLK$_{1133}$(+42)QQQEVAK(L)	95%	4,660	0.6422	779,9177	1.557,82	2	1,362
(K)QQQEVAK$_{1140}$(+42)LR(E)*	95%	2,559	0.5444	571,3196	1.140,62	2	-0,4438
(R)K$_{1178}$(+42)GmPIATPVFDGAK(E)	95%	3,851	0.6688	745,3899	1.488,77	2	0,1888
(K)ELLK$_{1200}$(+42)LGDLPTSGQIR(L)	95%	4,029	0.5342	841,4792	1.680,94	2	1,418
(R)STGSYSLVTQQPLGGK$_{1262}$(+42)AQFGGQR(F)	95%	4,335	0.5947	803,744	2.408,21	3	0,543

Lysine-acetylated β' peptides identified from glucose-exposed wild-type cells

Sequence	Prob	SEQUEST XCorr	SEQUEST ΔCn	Observed	Actual Mass	Charge	Delta PPM
(K)AQTK$_{13}$(+42)TEEFDAIK(I)*	95%	3,134	0.5511	711,8596	1.421,70	2	0,4564
(K)K$_{40}$(+42)PETINYR(T)	95%	1,857	0.3906	531,7829	1.061,55	2	0,5715
(R)GLATTIK$_{395}$(+42)AAK(K)	95%	2,728	0.654	508,3111	1.014,61	2	0,1017
(K)GEGmVLTGPK$_{531}$(+42)EAER(L)	95%	3,620	0.5413	766,3754	1.530,74	2	0,8169
(K)TSLK$_{570}$(+42)DTTVGR(A)	95%	2,799	0.4491	560,3032	1.118,59	2	-1,289
(R)SGASVGIDDmVIPEK$_{649}$(+42)K(H)	95%	3,297	0.5722	852,4306	1.702,85	2	0,9404
(R)AAAESSIQVK$_{953}$(+42)NK(G)	95%	3,437	0.5779	644,3492	1.286,68	2	0,6021
(K)GSIK$_{959}$(+42)LSNVK(S)	95%	2,737	0.5114	494,2947	986,5748	2	-1,35
(K)LSNVK$_{964}$(+42)SVVNSSGK(L)	95%	3,015	0.6372	680,8746	1.359,73	2	-0,8405
(R)NTELK$_{983}$(+42)LIDEFGR(T)	95%	3,479	0.5128	492,9273	1.475,76	3	-1,452
(R)LQGVK$_{1247}$(+42)INDK(H)	95%	1,808	0.2969	528,8063	1.055,60	2	0,2463

Table 4. Immunoprecipitated β and β′ proteins were separated by SDS-PAGE and β and β′ bands were digested with trypsin, as described (20). The resulting peptides were analyzed in a LTQ OrbitrapXL mass spectrometer, as described in the experi-

mental procedures section and analyzed using the Scaffold software. The table shows the Xcorr, DCn scores, charges, both the observed precursor m/z and actual peptide mass, and the mass deviation (delta ppm) of all 15 K-acetylated peptides detected for b and 11 Lys-acetylated peptides detected for β′ in the glucose-exposed samples.

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